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THE FORMATION OF THE EGG OF THE DOMESTIC FOWL[1]

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The hen's egg is a common material for the study of embryology, and for this reason its structure and formation have been described frequently in text books. The process of egg formation is also of particular interest to those engaged in fundamental investigations of the problems of market egg production. In the hen, the left gonad and left genital duct develop into the ovary and oviduct respectively, while the development of the corresponding organs on the right side is arrested at an early stage. The yolk of the egg, or ovum, is formed in the ovary, and the white, shell membranes, and shell are formed in the oviduct.

FORMATION OF THE OVUM. Since Marza and Marza (38) have recently reviewed the histology of yolk formation, the early stages of this process will not be discussed, but only the last stage, in which yellow yolk is formed.

Before sexual maturity and while the mature hen is out of production the ovary appears as a mass of immature follicles ranging in size from microscopic up to a limit which approaches 6 mm. in diameter as the hen approaches maturity. These follicles contain only the white yolk of the early stages of yolk formation, which differs markedly in composition from the yellow yolk of the last stage of formation (50, 38). As the hen approaches the period of egg production, certain of these follicles, one after another, enter the third stage of yolk formation (as described by Van Durme, 62) characterized by very rapid growth and the formation of yellow yolk. As rapidly as these reach maturity and are ovulated, others begin developing, so that there is present a series of from four to ten ova ranging in size from 6 mm. to the size of the mature yolk. The number of ova in this stage at a given time depends on a number of

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factors. If the individual ova are growing rapidly, if they are ovulated when still rather small or if the hen is laying at a low rate, the number will tend to be small. Conversely, slow growth, large size of the mature ovum, and high laying intensity are factors associated with a large number of ova in this stage of formation. By feeding sudan III, a fat soluble dye, Riddle (50, 51) found that when ova enter the third stage of formation the rate of growth suddenly increases about 25 times, and that the radius of an ovum in this stage increases about 2 mm. a day. Marza and Marza (38) and Stieve (59) believe that this transition occurs more slowly but agree with Riddle as to its magnitude.

Stieve investigated the rate of growth of ova by killing laying hens and measuring all the larger ova. He assumed that the difference in size between any two consecutive ova represented the amount one would grow in one or two days, depending on whether that particular bird usually laid daily or every other day. Riddle (50) measured the growth rate by feeding sudan III at regular intervals. Each feeding produced a layer of dyed yolk, and when the laid egg was hard boiled and sectioned, the distance between rings indicated the amount of growth between feedings. Warren and Conrad (unpublished) have refined this method slightly by injecting intravenously a colloidal suspension of the same dye. Their method has a number of advantages: 1, deposition of the dye begins immediately after injection; 2, deposition is not affected by the rate of absorption from the intestinal tract; 3, much smaller doses may be used, and 4, the dye may be administered more frequently without serious overlapping of the dyed layers. By the injection of dye at 12 hour intervals, it was found that the rate of growth is nearly uniform throughout the 24 hour day. The thickness of the layer of yolk deposited in a day was found to be about 2 mm. during the early part of the period under investigation, and to decrease gradually to about 0.7 mm. the last day before ovulation.

Little is known of the mechanism of formation of this yolk material. There are three general ways in which yolk might be formed. The materials of the yolk might be synthesized elsewhere, carried to the ovary in the blood and deposited in the yolk. They might be formed in the follicular epithelial cells, and secreted into the yolk. Finally, the yolk material might be synthesized in the yolk from nutrients reaching it through the follicular epithelium from the blood. Perhaps all three mechanisms have some part in yolk formation. Bartelmez (6) has shown that in the pigeon, after the formation of the zona radiata, a narrow radially striated zona just inside the follicular epithelium which,

according to Lillie (37), becomes the vitelline membrane of the yolk, the ovum is free to turn within the follicle, indicating the presence of a lymph space between the ovum and the follicular epithelium. In the pigeon (6) and in the hen (11) the zona radiata is formed before the ovum enters the last stage of yolk formation.

Holl (33) has described pores passing through the zona radiata and suggests that the nutrient enters the yolk through these. Riddle (50) suggests that the nutrients diffuse from the blood into the yolk where they are changed into yolk material. He believes that the peripheral layer of white yolk is an intermediate stage in the formation of yellow yolk. Riddle has pointed out that by no means all the yolk material may be considered to be synthesized by the follicular epithelial cells, since such a mechanism would require that these secrete a volume equal to their own every half hour. Marza and Marza (38) suggest that the function of the follicular epithelium is to control the composition of the materials going into the yolk by exerting a selective permeability. As support for this viewpoint they cite Bialaszewicz (8) who has shown by ultrafiltration studies that the potassium ion concentration is much greater and the sodium ion concentration much less in yolk than in blood serum. Roepke and Hughes (54), and Roepke and Bushnell (53) found that the blood of actively laying hens contains vitellin, which suggests that it might be synthesized in some other part of the body and carried to the ovary, where it is transferred to the yolk material.

The transportation of large amounts of lipids from the blood stream into the ovum represents a problem as yet unsolved. Bartelmez (6) has suggested the presence of a lymph space between the follicular epithelium and the vitelline membrane. That lipids are transported across this space rather than synthesized from water soluble materials within the ovum follows from the fact that typically fat soluble compounds such as the xanthophylls and fat soluble dyes are deposited in the ovum when fed in the diet. Cruickshank (21) has shown that the proportion of the different fatty acids found in the yolk can be influenced by the feed. According to Lillie (37), Meckel v. Hemsback in 1851 described alternate white and yellow layers in the yellow yolk of the hen's egg. A number of more recent investigators have described these layers. Riddle (50) has shown that they correspond to a daily variation in yolk formation. According to him the white layers are incompletely formed yellow yolk, left as the mark caused by a deficiency of yolk forming nutrients, which occurs between 1 and 5 a.m. Certain other investigators have been unable to find these layers, and Balbiani (5)

suggested that they are the same as yellow yolk, but contain less yellow coloring matter than the adjacent layers. That this is quite probably true is indicated by the fact that Conrad and Warren (unpublished) were unable to find these layers in eggs from hens fed a uniform mash *ad lib*, but did find them in some farm eggs, and could produce them by various dietary regimes. They were able to produce eggs showing these alternate layers simply by restricting the feeding period to 6 hours a day or by feeding a mash poor in the egg coloring xanthophylls supplemented daily by a small amount of yellow corn or green grass.

It has been shown by Palmer (42) that the yellow color of the yolk is due to carotinoid pigments, principally xanthophylls, ingested in the feed. The ordinary pigments of the yolk are lutein and zeaxanthin (36). Cryptoxanthin (29) and capsanthin (12) may also be deposited in the yolk.

The yolk is formed in continuous, concentric layers except in the region of the blastoderm, or germ spot, where no yellow yolk is deposited. Since the blastoderm remains on the surface of the ovum, the result is that a column of the latebra, the white yolk formed in the early stages of yolk formations, connects the blastoderm with the main bulk of the latebra in the center of the ovum. More yellow yolk is deposited in the hemisphere opposite the blastoderm than in the hemisphere containing it. This causes the latebra mass to be displaced slightly from the center of the yolk, and probably accounts for the fact that the yolk tends to float with the blastoderm on top.

Ovulation. The mechanism of ovulation has been studied by Phillips and Warren (47) on anesthetized hens. Their evidence indicates that the contraction of muscle fibres in the follicular wall causes it to split along the stigma, a narrow non-vascular area opposite the stalk. The source of the stimulus responsible for this muscular contraction has not been established although it is generally assumed to be endocrine in nature. Several reports have appeared in the literature which indicate that follicle stimulating hormones of hypophyseal and pregnancy urine origin will reduce the interval between successive ovipositions (48). A reduction in interval of one hour or the laying of a single complete egg followed by two partially formed ones within a period of 41 hours, does not necessarily indicate a change in the rhythm of ovulation. On the contrary, no change in ovulatory rhythm need be assumed if the two eggs lacking a complete complement of egg envelopes were prematurely expelled. Subcutaneous, intraperitoneal, and intravenous injections of antuitrin or prolan given in large single dosages or in

smaller quantities over a period of five days have failed to induce any change in the rhythm of ovulation (Scott, unpublished data).

That ovulation is under some very positive control seems obvious from the regularity with which a given hen lays. Warren and Scott (63, 64) have shown that ovulation usually follows the laying of the previous egg within about 30 minutes except when the previous egg is laid late in the afternoon, in which case ovulation is delayed until early the following morning. The time spent in the oviduct by each egg is quite uniform and characteristic of the hen. If the interval between eggs is 26 hours for a certain hen, and she begins a clutch by ovulating at 6 a.m., she will lay that egg and ovulate the next about 8 a.m. the next day. She will then lay at 10 a.m., 12 noon, and 2 p.m. on successive days and the following day will lay at 4 p.m., but will not ovulate. Thus the clutch is terminated with the fifth egg, and none is laid the following day, although the first egg of the next clutch is ovulated early in the morning of that day. It seems certain that ovulation is not a result of laying, since ovulation occurs at the regular time even when the previous egg is prematurely removed from the uterus (63). Perhaps both are a result of the same stimulus. It has been observed that a hen known to be ovulating into the body cavity, and hence not laying, will in some cases be found in the trapnest as regularly as though she were laying (44).

The effect of light on laying, and hence on ovulation, is fairly well known. The failure of a hen to ovulate late in the afternoon seems definitely associated with approaching night. If a hen is subjected to continuous, uniform illumination, the tendency to lay in clutches is reduced, and ovulation or oviposition may occur at any hour of the day or night (65). The response to natural daylight supplemented by artificial light during the night does not differ from that observed under natural conditions. By excluding all natural light and supplying artificial light only during natural night, hens can be induced to lay only at night, during their artificial "daytime."

FORMATION OF THE EGG WHITE. Warren and Scott (63) have shown that, on the average, an ovum enters the oviduct about 15 minutes after ovulation, passes through the infundibulum or funnel in 18 minutes, spends 3 hours in traversing the magnum, or albumen secreting portion of the oviduct, 1 hour in the isthmus, and the remainder of the interval, usually 20 to 24 hours, in the uterus.

The method by which the egg is carried through the oviduct has not been demonstrated conclusively. Cushny (24) and Harper (31)

state that in the hen and pigeon respectively, the egg is propelled entirely by ciliary action after it has passed through the infundibulum. Curtis (23), however, believes that the egg is propelled by the muscles of the wall of the oviduct. Warren and Scott (63) report considerable peristaltic action in the oviduct above and below the egg, and suggest the possibility that the contractions are present, but obscured by the distention of the oviduct, in the region of the egg.

It was shown by Tarchanoff (61), and has been confirmed by several subsequent workers, that if an artificial ovum, such as a small rubber ball, is inserted into the infundibulum, it will become surrounded by all the normal egg structures as it passes through the oviduct. The ovum, then, must supply only a mechanical stimulus to the oviduct. It is possible that the endocrine mechanism connected with laying and ovulation also plays a part in preparing the oviduct to receive the ovum and secrete the white. Several failures of this experiment have been reported, but in this laboratory it has been almost universally successful when the artificial ovum has been inserted at the time of natural ovulation.

In the laid egg, 4 layers of white have been observed: 1, the inner thick or chalaziferous layer, which is quite gelatinous, and is extended toward the poles of the egg to form the chalazae, the white cord-like structures which are attached to the yolk of a fresh egg; 2, the inner thin white, composed of 5 to 10 cc. of fluid; 3, the thick or "viscous" white, consisting of about 20 cc. of gelatinous material, and 4, the outer thin white, another layer of fluid. Surface (60) suggested that the chalaziferous layer of white, and the chalazae are secreted in the infundibulum. Since it has been shown that the chalaziferous material is not deposited as such, but is formed from the white secreted by the magnum, and because of great structural difference between the funnel and the magnum, it seems quite probable that none of the white is secreted in this part of the oviduct. In the magnum, white is secreted around the yolk as a very dense, apparently homogeneous gel (30). Structurally, this gel consists of a network of mucin fibers (17) enclosing a solution of the other proteins of the white, ovalbumin, conalbumin, ovoglobulin, and ovomucoid (32).

It was observed by Curtis (23) that the shape of the egg is determined to some extent in the magnum. If a sharp contrast in shape exists between the two poles of fully formed eggs laid by a given hen, the same general outline can be observed in a partially formed egg removed from the lower third of the magnum of the same bird. Asmundson and

Jervis (4) altered egg shape by resection of the isthmus and concluded that the uterus plays a minor rôle in determining egg shape and "normally exerts little other effect than to preserve the shape that the egg has when it leaves the isthmus." By noting the shape and position of many eggs removed at necropsy from various levels of the oviduct Scott and Warren (unpublished data) concluded that 1, the isthmus normally does little more than to preserve the shape established in the magnum, and 2, the small end of the egg if discernible is always directed caudalward in all divisions anterior to the uterus.

It has long been supposed that the chalazae and chalaziferous layer were formed in the upper part of the oviduct by a special secretory mechanism. This view was expressed as recently as 1936 by Richardson, although Hansen (30) had reported that the chalazae did not appear until after the egg had reached the uterus.

Conrad and Phillips (19) have reported that the inner thin and chalaziferous layers, as well as the chalazae, arise simultaneously from the gel by the mechanical segregation of its mucin fibres. According to them, the innermost part of the gel becomes partially liquefied before the egg reaches the uterus, perhaps due to the mechanical action associated with the propulsion of the egg through the magnum. This leaves the yolk free to turn within the white. The egg is rotated about its long axis while it is in the uterus, and since the yolk always tends to float with the blastoderm up, the white is rotated about the yolk. This action wraps the mucin fibers around the yolk and twists them at the ends to form the chalazae. Removal of the mucin fibers from the inner layer of gel liquefies it, and it becomes the inner thin white.

While the egg is in the uterus, the volume of white is nearly doubled (43) by the addition of the uterine secretion through the membrane. Pearl and Curtis, and others, postulated that protein was also added with this fluid. Scott, Hughes, and Warren (55) have shown, however, that this entails little or no increase in the protein content of the white. Beadle, Conrad, and Scott (7) have analyzed the uterine fluid and calculated the composition of the uterine secretion. According to them, the fluid added to the egg is essentially a solution of inorganic salts, especially rich in potassium and bicarbonate ions. The influx of the fluid is due to osmosis—the shell membranes, although somewhat permeable to the proteins of the egg white, retard their diffusion sufficiently to produce a definite osmotic pressure difference across the membrane. A membranous egg placed in artificial uterine fluid will swell to at least twice the normal egg size. Since there is always some fluid in the

uterus, it seems highly probable that the influx of this fluid into the egg must ordinarily be stopped by the decrease in permeability of the membranes due to the deposition of shell.

When this solution enters the egg, it extracts part of the soluble protein from the gel, leaving the gel with less of the soluble proteins and relatively more mucin than before. The soluble proteins will diffuse out into the solution, and water will diffuse into the gel and inner thin white until the water and soluble protein concentrations are the same in all three of these layers. Since mucin makes up only 2 per cent of the total protein of the egg white (56), we might expect the total solids content of all three fractions to be very nearly the same at equilibrium. Conrad, Hughes, and Scott (unpublished) have followed the distribution of water in the egg during this stage of formation by determining total solids directly. The difference in total solids between the inner and and outer layers of white reaches a maximum about 10 hours after ovulation (at which time the values are about 20 per cent and 4 per cent respectively). These values approach each other and become about 12 per cent and 10 per cent respectively at the time of laying. Almquist and Lorenz (2), in studying changes in the solids content of laid eggs found that this distribution of water and solids practically had reached equilibrium by the fifth day after laying.

THE SHELL MEMBRANES. As the egg passes through the isthmus, the shell membranes are formed around the gel which constitutes the white at this stage. When first formed, the membranes fit the egg tightly, and have the appearance of having been formed by the transformation of the outer layer of gel. Therefore, they have the shape of the egg white gel. Shortly after formation, the membranes seem to stretch, and by the time the egg reaches the uterus, enclose the egg contents quite loosely. The shell membranes are usually described as being made up of two layers (16). It has been shown that they consist of a network of microscopic fibers (41) having the properties of a keratin (14). Giersberg (28) has described the formation of these fibers. According to him, the material is secreted in the form of droplets which coalesce into fibers.

THE FORMATION OF THE SHELL. Warren and Scott (64) have shown that the egg remains in the uterus 20 to 24 hours of the interval between ovulation and laying. Unpublished work at this station and at the Illinois Agricultural Experiment Station indicates that shell deposition commences practically as soon as the egg reaches the uterus. No work has yet been done which would indicate when shell deposition

ceases, and it possibly may continue until oviposition although the shell is usually quite rigid 13 hours before laying. Pearl and Surface (46) have shown that the secretion of shell is stimulated mechanically. They anastomosed the upper end of the uterus to the lower end of the intestine, and found that fecal matter passing through the uterus was enclosed in shell material.

During the time the egg is in the uterus, approximately 5 grams of nearly pure calcium carbonate are deposited on the shell membranes as the egg shell. The structure of the shell has been described by Stewart (58) in a review. Next to the shell membranes is a layer of knob-like calcite crystals, or mammilla. These mammilla are the first part of the shell to be formed and appear to compose most of the shell of eggs removed from the oviduct 10 hours or less after ovulation. Outside the layer of mammilla is the so-called spongy layer, composed of very small crystals of calcite. This layer constitutes about two-thirds of the total thickness of the shell.

Besides its mineral content, the shell contains some protein, usually described as matrix or cuticle. Almquist (1) has described the cuticle as being a collagen-like protein. Part of the protein usually found in the shell must, structurally at least, be part of the shell membranes. Stewart (58) has observed that fibers from the shell membranes penetrate into the shell, and stay with the shell when the membranes are peeled off. Richardson (49) was unable to find any protein secretion of the uterus by histological means. Since the cuticle seems to be formed in and around the particles of shell, it probably is not deposited in the vagina as has been suggested by Surface (60), because the egg remains in the vagina only a few seconds at most. An undemonstrated possibility which suggests itself is that the cuticle is formed by soluble proteins which diffuse out of the egg white through the shell membrane and are rendered insoluble by "surface denaturation." It has been demonstrated that egg albumin can be denatured by adsorption on a surface. The calcite crystals present during the formation of the shell certainly provide an abundance of surface.

Furreg (27) has reported that the porphorin of the cuticle which fluoresces in ultra violet light is deposited in the uterus. Unfortunately he has identified the fluorescence with the cuticle, and supposes that the disappearance of the fluorescence upon exposure to light indicates the destruction of the cuticle, although the protein remains after this treatment.

It is probable that, as has been suggested by Stewart, at least the

large pores of the shell are formed by fibers from the shell membranes which project through the shell. When the shell dries, these shrink, leaving a pore opening through the shell. It has been observed that ordinarily the large end of the egg has many more large pores than the small end. If an egg is removed from the oviduct just after the membranes have been formed, it can be seen that there are many more loose fibers on the blunt end, where the membranes are formed last.

According to Richardson (49), calcium carbonate is secreted directly on the shell membrane by uterine cells in contact with it. Those cells not in contact with the shell membranes or shell, due to the folding of the uterine epithelium, do not show the high concentration of calcium ash found in the cells which are in contact with it. That the calcium carbonate can not simply be secreted in solution, from which it crystallizes on the egg, can be shown by calculating the volume of secretion necessary. Under the conditions found in the uterine fluid (7) it would take 10 liters of secretion saturated with calcium carbonate to carry the 5 grams laid down in 20 hours. Furthermore, most of the shell is deposited after the eighth hour following ovulation, at which time the total fluid content of the uterus is not increasing appreciably, although there remains the possibility of the fluids being secreted into the uterus and, after giving up part of its calcium content, being again absorbed by another part of the uterine wall. Probably calcium and bicarbonate ions are secreted into the uterine lumen without the secretion of appreciable amounts of fluid, or perhaps the uterine cells actually secrete a suspension of solid calcium carbonate crystals.

The avian female, and particularly the domestic hen, is unique because of the large amounts of calcium that must be mobilized and transported in a short time during egg shell formation.

Hughes, Titus, and Smits (34) have shown that the blood serum of laying hens contains 2 to 3 times as much calcium as non-laying hens or cocks. Correll and Hughes (20) have shown that this increased amount of calcium is accounted for in the non-ultrafiltrable fraction, the amount of filtrable calcium being the same in the serum of laying and non-laying birds.

Although all the calcium of the shell comes originally from the diet, it seems that even under favorable dietary conditions, body calcium frequently is mobilized for shell formation. Morgan and Mitchell (40) have found that during the early laying period a pullet is in negative calcium balance, but the balance frequently becomes positive later on without any cessation of laying. Deobald, Lease, Hart and Halpin

(25) have shown that 10 per cent of the bone calcium can be available for shell formation. Keyes and Potter (35) have shown that laying female pigeons exhibit ossification of the bone marrow while males and non-laying females do not. It has been shown by Deobald *et al.* (25) and Feinberg, Hughes and Scott (26) that the blood serum calcium of the laying hen is quite constant throughout the cycle of egg formation. It follows from this, that as calcium is removed by the uterus, it is replaced readily by the bone reserves or from the alimentary tract, no doubt from both under normal conditions.

Common (18) found that the phosphorus content of the excreta of a hen increases during the period of shell formation, and attributed this to the liberation of bone phosphorus when calcium is removed from the bone to form shell. Feinberg, Hughes, and Scott (26) found an increase of 75 per cent in blood serum phosphate when an egg was in the uterus, whereas no similar diurnal change was observed in non-laying hens.

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APPLICATION OF THE LAW OF CHEMICAL EQUILIBRIUM (LAW OF MASS ACTION) TO BIOLOGICAL PROBLEMS

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The concept of balanced chemical reactions, introduced by Wenzel in 1777[1] and made more exact by Berthollet in 1801, was put into the quantitatively useful form of the law of mass action by Guldberg and Waage in 1867. In 1877 van't Hoff showed how this law could be derived from the principles of thermodynamics, without introducing the vague idea of chemical force, and in 1887 Arrhenius applied the same law to the dissociation of electrolytes in solution.

The earliest applications of the law of chemical equilibrium, which was originally derived from the law of mass action, to physiological problems were that of Hüfner (1890) to the dissociation of oxyhemoglobin, and that of L. J. Henderson (1908), characterized by Peters and Van Slyke (1931) as "the first unified explanation of the physiological and physicochemical mechanisms by which the body maintains its normal acid-base balance."

This review will attempt to set forth the concepts essential to a working knowledge of the law of chemical equilibrium, and to illustrate the adaptability of this law to specific problems, rather than to assemble the many important though unrelated contributions which have made use of it. The equally important applications of the law of mass action to the kinetics of chemical reactions will be considered only in so far as they have been applied to physiological problems studied also by the law of chemical equilibrium.

THE LAW OF CHEMICAL EQUILIBRIUM[2]. The law of mass action, as stated by Guldberg and Waage, is essentially that the rate at which a substance reacts is proportional to its "active mass," and that the velocity of a chemical reaction is proportional, at any moment, to the

[1] For a full exposition of the history of chemical affinity and chemical equilibrium see M. M. Pattison Muir (1909).

[2] For an extended development of the law of chemical equilibrium in homogeneous systems see Edgar (1931). For a simplified derivation from kinetics see Hogness and Johnson (1937) or Stieglitz (1911).

product of the active masses of the substances reacting, and to a constant characteristic of the particular reaction (and of the temperature). Guldberg and Waage also pointed out clearly the general reversibility of chemical reactions, and derived from their consideration of the kinetics of such reactions the law of chemical equilibrium.

The more generally useful, and in some instances indispensable, derivation of the law of chemical equilibrium is that from the principles of thermodynamics, first applied to this law by van't Hoff. The derivation from the principles of kinetics, as applied by Guldberg and Waage, subsequently modified by the introduction of the activity concept, is adequate for the interpretation of the relationships of concentrations of reacting substances at equilibrium, and does not require familiarity with thermodynamics. In this review the effort is made to present the subject, wherever possible, without the introduction of thermodynamics, reserving the latter for those special uses of the law of chemical equilibrium which require its application.

The activity concept. In either case, use of the activity concept, although not always attainable in actual practice, is essential to an adequate statement of the law of chemical equilibrium and to definition of the limitations put upon this law when practical considerations prevent its full application. The term "active mass" as used by Guldberg and Waage had an indefinite meaning,[3] but they pointed out that "molecular concentration" apparently could be substituted for it. The modern substitution of thermodynamically defined "activity" for the vague "active mass" makes possible a theoretically comprehensive and what Edgar (1931) calls a universally valid statement of the law of chemical equilibrium.

A full discussion of the concept of activity would be beyond the scope of this review. If it is desired to find an equivalent for the term, it may be thought of as the "apparent concentration" or "effective concentration," although these terms add nothing to the "active mass" of Guldberg and Waage. The activity of a "perfect gas" is equal to its pressure, and for any actual gas the activity approaches the pressure as the gas becomes dilute. In the case of aqueous solutions the activity of a solute approaches its molality as the solution becomes dilute. The activity of a solute is in part dependent upon properties of the solute

[3] Guldberg and Waage defined the "active mass" of a substance as the quantity of it within "the sphere of attraction" or "the sphere of action." As they could not determine the absolute sizes of such spheres of action, they chose an arbitrary volume.

itself and of the solvent, and in part upon the concentration of the solute itself and of other solutes. This effect of the concentration of a solution upon the activities of its solutes has been found in the case of electrolytes to depend more particularly upon the total "ionic strength" (Lewis and Randall, 1923) which is a function of the concentration and of the valences of ions in the solution.

The relationship between the molal concentration of a solute, under any particular conditions, and its activity is expressed by a factor known as the activity coefficient. The theory of Debye and Hückel[4] attempts to account for the effects of various properties of solutes and solvents upon the activity coefficient, and for the empirical observation that the logarithm of the activity coefficient has a linear relationship to the square root of the ionic strength.

Dissociation of electrolytes. The concept of activity, arising in part out of efforts to explain the behavior of electrolytes in solution, has greatly modified the theory of their dissociation, to which the law of chemical equilibrium was first applied by Arrhenius. There is now almost universal acceptance of the belief that the so-called strong electrolytes are completely ionized in aqueous solution, the effect of increasing concentration upon conductivity and other properties of such solutions now being attributed to changes in the activities of the ions. Obviously, then, the law of chemical equilibrium can not be used as a measure of the degree of dissociation of strong electrolytes. On the other hand, the weak electrolytes, such as the weak acids and weak bases, are regarded as only partially ionized and the law of chemical equilibrium is applicable to their dissociation. There appear to be certain exceptions, some of them of biological interest, to the general rule that salts are strong electrolytes. Calcium and magnesium form salts with many weak acids, including proteins, whose behavior in solution is better explained by the assumption of partial dissociation than by application of the activity concept alone.

The law of chemical equilibrium from the standpoint of kinetics. As stated by Guldberg and Waage, but modified by introduction of the activity concept, the velocity of the reaction

$$A + B \rightarrow C + D \tag{1}$$

may be expressed by the equation

$$v_1 = k_1 \times a_A \times a_B \tag{2}$$

[4] For full treatment of the Debye-Hückel theory see Taylor (1931).

in which v_1 is the velocity of the reaction, k_1 is the velocity proportionality constant characteristic of this reaction, and a_A and a_B are the activities of A and B.

For the reverse reaction

$$A + B \leftarrow C + D \tag{3}$$

$$v_2 = k_2 \times a_C \times a_D \tag{4}$$

and for the reversible reaction

$$A + B \rightleftharpoons C + D \tag{5}$$

equilibrium, being by definition the condition at which the velocities of the two opposing reactions are equal, is defined by the equation

$$\frac{a_C \times a_D}{a_A \times a_B} = \frac{k_1}{k_2} = K \quad \text{(equilibrium constant)} \tag{6}$$

When one component appears more than once in a reaction, as

$$A + 2B \rightarrow C + D \tag{7}$$

$$v_1 = k_1 \times a_A \times a_B^2 \tag{8}$$

and for equilibrium

$$\frac{a_C \times a_D}{a_A \times a_B^2} = \frac{k_1}{k_2} = K \tag{9}$$

which may be expanded, for the general case

$$aA + bB + \cdots \rightleftharpoons cC + dD + \cdots \tag{10}$$

to

$$\frac{a_C^c \times a_D^d \times \cdots}{a_A^a \times a_B^b \times \cdots} = K \tag{11}$$

which is the universally valid statement of the law of chemical equilibrium.

In the case of equilibria of biological interest, equation 11 is rarely applicable for the reason that the activities of the various components can not be arrived at, the chief obstacles being uncertainty concerning both the activities of proteins and other complex substances in biological fluids, and the effects of these substances upon the activities of other

solutes. Consequently it is usually necessary to make the simplifying assumption, as did Guldberg and Waage, that activities are proportional to molality and write equation 11 as

$$\frac{[C]^c \times [D]^d \times \cdots}{[A]^a \times [B]^b \times \cdots} = K' \tag{12}$$

in which the brackets [ ] represent concentrations in terms of moles per kilogram of water and K' represents an apparent equilibrium constant.

Equation 12 may be transformed to the universally valid equation 11 by the introduction of factors which represent activity coefficients, thus

$$a_A = f[A] \tag{13}$$

The use of equation 12, by omitting activity coefficients of the various components, implies the assumption that these coefficients remain constant. This condition is approximated in the case of dilute solutions of weak electrolytes, at constant ionic strength, which makes the use of equation 12 valid for most purposes under these limiting conditions. These limitations, to which further reference will be made, must of course be kept in mind whenever the attempt to use equation 12 is made.

The law of chemical equilibrium from the standpoint of thermodynamics.[5] The general criterion of equilibrium is that the free energy of the system, or that part of the energy which may be made to do useful work, remains unchanged. Thus, for equilibrium

$$\Delta F = 0 \tag{14}$$

If we again consider the reaction

$$aA + bB \rightleftharpoons cC + dD \tag{10}$$

as applied to a system of perfect gases, we may imagine two different equilibrium systems I and II, at pressures P_1 and P_2, with volumes so large that removal of small quantities of the reacting components does not alter the composition of the systems appreciably. From system I we remove a mols of A and b mols of B, at the partial pressures P_{1_A} and P_{1_B}. For this step $\Delta F = 0$, since the condition of equilibrium is not measurably disturbed. If the pressures of the gases withdrawn are

[5] The derivations which follow are given in greater detail by Edgar (1931).

changed to P_{2_A} and P_{2_B}, at which they exist in system II, the free energy change, from the gas laws, is

$$\Delta F_1 = aRT\ln\frac{P_{2_A}}{P_{1_A}} + bRT\ln\frac{P_{2_B}}{P_{1_B}} \tag{15}$$

in which R is the gas constant, T the absolute temperature and ln the natural logarithm.

We may now introduce the gases into system II, and since this does not measurably disturb the equilibrium, again $\Delta F = 0$ for this step.

If the process is simultaneously carried out in the reverse direction

$$\Delta F_2 = cRT\ln\frac{P_{1_C}}{P_{2_C}} + dRT\ln\frac{P_{1_D}}{P_{2_D}} \tag{16}$$

and at the end the composition of each system is what it was at the beginning, therefore

$$\Delta F = \Delta F_1 + \Delta F_2 = 0 \tag{17}$$

and from equations 15 and 16, by rearrangement

$$RT\ln\frac{P^c_{2_C}\times P^d_{2_D}}{P^a_{2_A}\times P^b_{2_B}} = RT\ln\frac{P^c_{1_C}\times P^d_{1_D}}{P^a_{1_A}\times P^b_{1_B}} \tag{18}$$

and

$$\frac{P^c_{2_C}\times P^d_{2_D}}{P^a_{2_A}\times P^b_{2_B}} = \frac{P^c_{1_C}\times P^d_{1_D}}{P^a_{1_A}\times P^b_{1_B}} \tag{19}$$

or since these were any two sets of equilibrium pressures

$$\frac{P^c_C\times P^d_D}{P^a_A\times P^b_B} = K \tag{20}$$

which is identical, for a system of perfect gases, with equation 11, and which may equally well be derived for substances in solution, in the form of equation 11.

By a similar process, and considering again the reaction

$$aA + bB \rightleftharpoons cC + dD \tag{10}$$

for a system of perfect gases the following expression may be derived for the free energy change for the reaction, letting $P_{A'}$ and $P_{B'}$ be the initial partial pressures of A and B, $P_{C'}$ and $P_{D'}$ the final partial pressures of C and D, and P_A, P_B, P_C and P_D the partial pressures of the four gases at equilibrium.

$$\Delta F = RT\ln \frac{P_{C'}^{e} \times P_{D'}^{d}}{P_{A'}^{a} \times P_{B'}^{d}} - RT\ln \frac{P_{C}^{e} \times P_{D}^{d}}{P_{A}^{a} \times P_{B}^{b}} \quad (21)$$

and by substitution of equation 20

$$\Delta F = RT\ln \frac{P_{C'}^{e} \times P_{D'}^{d}}{P_{A'}^{a} \times P_{B'}^{b}} - RT\ln K \quad (22)$$

The free energy increase of the reaction is thus seen to be determined, at constant temperature, by the equilibrium constant and by the initial and final partial pressures of the reacting species. If $P_{A'}$, $P_{B'}$, $P_{C'}$ and $P_{D'}$ are all equal to unity, equation 22 becomes

$$\Delta F = -RT\ln K \quad (23)$$

This derivation has the advantage that it relates the equilibrium constant to the free energy change of the reaction, a quantity which is of especial interest in the study of the energy metabolism of biological organisms. The free energy change may also be calculated from thermochemical data, and in the case of electromotively active systems from electrode potentials. Consequently data obtained in a variety of ways may be expressed in terms of the equilibrium constant, of the change in free energy, or of electrode potentials, according to the requirements of the particular problem under investigation.

Dissociation of oxyhemoglobin. The first attempt at application of the law of chemical equilibrium to a biochemical problem appears to have been that of Hüfner (1890) to the dissociation of oxyhemoglobin. It is of interest that this first and apparently obvious application has not yet been completely successful in interpreting the equilibrium between oxygen and hemoglobin.

Making the assumption that one molecule of oxygen combines with one molecule of hemoglobin, and hence from the equation

$$HbO_2 \rightleftharpoons Hb + O_2 \quad (24)$$

Hüfner wrote the simple mass-law equation

$$\frac{[Hb] \times [O_2]}{[HbO_2]} = K^{6} \quad (25)$$

[6] Throughout this review we have followed the convention of placing the dissociating substance on the left hand side of the stoichiometric equations and in the denominator of mass-law equations. For this reason mass-law equations, as compared with those originally published, will at times be found inverted.

This equation was found by Barcroft (1914) to agree fairly well with experimental data in the case of dialyzed (salt-free) hemoglobin. It was found very early, however, that equation 25 was not applicable to the dissociation of oxyhemoglobin in blood, or in solutions containing salts. The earlier history of this subject is extensively reviewed by Barcroft.

Aggregation hypothesis. Hill (1910), in an attempt to explain the behavior of hemoglobin in the presence of salts, and still assuming that one molecule of Hb in distilled water combined with one molecule of O_2, amplified equation 24 with the object of ascertaining the shapes of the dissociation curves which would be obtained on the assumption that the molecules of hemoglobin fall into aggregates as the result of the addition of salts. He reasoned that if on the average there were n molecules of Hb in each aggregate, and if one molecule of Hb combined with one molecule of O_2, equation 24 would become

$$Hb_n\,(O_2)_n \rightleftharpoons Hb_n + nO_2 \tag{26}$$

and making the assumption that each aggregate of Hb_n behaved from the standpoint of mass action as though it were one molecule, equation 25 would become

$$\frac{[Hb_n] \times [O_2]^n}{[Hb_n(O_2)_n]} = K \tag{27}$$

or more simply

$$\frac{[Hb] \times [O_2]^n}{[HbO_2]} = K \quad \text{(Hill-Barcroft equation)} \tag{28}$$

The value for n in this equation was found to be nearly constant at 2.5 in the presence of salts, the value for K varying with different species and to some extent with different individuals, and as later found (Henderson, 1920) with the CO_2 tension, or, more properly, with the pH.

The finding of a value of 2.5 for n in equation 28 was interpreted as supporting the aggregation hypothesis, which received further support from the measurement of the heat of reaction of hemoglobin and oxygen by Brown and Hill (1923). Later work, however, by other methods, led to general acceptance of a molecular weight for hemoglobin corresponding to a combination of 4 molecules of O_2 with each molecule of hemoglobin. Simple application of the law of chemical equilibrium to the larger molecular weight would involve rejection of the aggregation hypothesis and the writing of equation 24 as

$$HB(O_2)_4 \rightleftharpoons HB + 4O_2{}^{7} \tag{29}$$

from which equation 25 would become

$$\frac{[HB] \times [O_2]^4}{[HB(O_2)_4]} = K \tag{30}$$

from which it is seen that the value of n in equations 27 and 28 should be 4 instead of 2.5 for this simple treatment to be applicable, and that equation 30 should also hold for solutions of dialyzed hemoglobin.

Intermediate compound formation. Adair (1925) and Ferry and Green (1929) attempted to explain these discrepancies by a theory of intermediate compound formation, a possibility also considered by Barcroft. This theory, which is consistent with the experimental data, but for which no satisfactory proof is available, owing to the number of constants involved, assumes that HB, in the presence of O_2, forms the series HBO_2, $HB(O_2)_2$, $HB(O_2)_3$, $HB(O_2)_4$, each with a different dissociation constant. Adair also attempted to reconcile the apparent difference in behavior of hemoglobin in the presence and in the absence of salts by introducing corrections for the change in acid strength of hemoglobin on oxygenation, and showed also that the thermal data of Brown and Hill are consistent with the hypothesis of intermediate compound formation.

Sidwell, Munch, Barron, and Hogness (1938) have recently reviewed the sources of difficulty in analyzing the equilibrium between hemoglobin and oxygen, and have called attention to the necessity of re-study of the effects of electrolytes upon this equilibrium. They present data concerning the inhibition of oxygenation of dialyzed hemoglobin on addition of varying amounts of different salts, and after consideration of ionic strength effects conclude that in some cases the inhibiting effect is very probably due to the direct combination of anions with hemoglobin, a possibility also postulated by Roughton (1935). They also point out that bicarbonate has the greatest effect of the salts studied, and that a direct combination of HCO_3^- ions with hemoglobin may be of physiological importance by influencing the uptake and output of oxygen by the blood. Their experiments were performed at the iso-

[7] Even though it is now believed that one molecule of hemoglobin combines with 4 molecules of O_2 the symbol HbO_2 persists in the literature. We shall continue to employ Hb to indicate the O_2 combining groups of hemoglobin, each corresponding to one atom of iron, and HB to indicate the molecule of hemoglobin. By this notation HB = Hb_4.

electric point of hemoglobin, thus minimizing the influence of carbamino compounds with CO_2 upon the oxygenation of hemoglobin (Stadie and O'Brien, 1937).

The history of the problem of the dissociation of oxyhemoglobin is in part the history of the discovery of additional properties of hemoglobin. To Hüfner's first simple conception of the equilibrium between hemoglobin and oxygen there have had to be added the influences of electrolytes, of the variable acid strength of hemoglobin with oxygenation, and of the carbamino compounds with CO_2. Resolution of the difficulties in applying the law of chemical equilibrium to this problem will perhaps serve as an indication that the major properties of hemoglobin have been recognized.

THE ACID-BASE BALANCE. The law of chemical equilibrium, with special reference to the dissociation of carbonic acid, was first applied to the study of the acid-base balance by L. J. Henderson (1908), and modern treatment of the subject is a direct derivation of this application.

Carbonic acid dissociates in two steps, to which the following mass-law equations apply

$$\frac{a_{H^+} \times a_{HCO_3^-}}{a_{H_2CO_3}} = K_{1\,H_2CO_3} \tag{31}$$

$$\frac{a_{H^+} \times a_{CO_3^=}}{a_{HCO_3^-}} = K_{2\,H_2CO_3} \tag{32}$$

The second dissociation does not yield quantitatively significant amounts of $CO_3^=$ below pH 7.8, and has but little biological application.

Henderson pointed out that in a mixture of bicarbonate and dissolved CO_2, when the concentration of $BHCO_3$ is of the order of magnitude of that present in plasma and exceeds 10 per cent of that of CO_2, the HCO_3^- ions derived from H_2CO_3 are negligible in comparison with those from $BHCO_3$. Moreover, in accordance with the assumption, current at the time, that $BHCO_3$ was only partially dissociated, he introduced a factor to indicate the degree of dissociation, and wrote

$$\frac{[H^+] \times f[BHCO_3]}{[H_2CO_3]} = K \tag{33}$$

or letting $K' = K/f$

$$\frac{[H^+] \times [BHCO_3]}{[H_2CO_3]} = K' \quad \text{(apparent dissociation constant)} \tag{34}$$

For convenience Hasselbalch (1916–17) put this equation into the logarithmic form

$$\mathrm{pH} = \mathrm{p}K' + \log \frac{[\mathrm{BHCO_3}]}{[\mathrm{H_2CO_3}]} \tag{35}$$

or the classical Henderson-Hasselbalch equation, in which the symbol $\mathrm{p} = -\log$.

Certain limitations are imposed upon the applications of equation 35, chiefly by the limitations of the analytical data available. In general, the quantities which may be measured or estimated being pH, total CO_2, and dissolved CO_2 (which includes H_2CO_3) this equation, in practice, assumes the form

$$\mathrm{pH} = \mathrm{p}K'_{\mathrm{CO_2}} + \log \frac{[\text{total } \mathrm{CO_2} - \text{dissolved } \mathrm{CO_2}]}{[\text{dissolved } \mathrm{CO_2}]} \tag{36}$$

which satisfactorily represents the conditions at constant ionic strength in systems containing CO_2 only as HCO_3^-, dissolved CO_2, and H_2CO_3. There are many observations to the effect that this equation may be applied, without further correction, to serum or plasma in equilibrium with CO_2. The applications of this equation are extensively reviewed by Peters and Van Slyke, and the newer literature by Sendroy (1938).

Hydration of CO_2. When CO_2 is dissolved in water the reaction

$$\mathrm{CO_2} + \mathrm{H_2O} \rightleftharpoons \mathrm{H_2CO_3} \tag{37}$$

occurs. The earliest determinations of the dissociation constant of carbonic acid (Walker and Cormack) implied the assumption that this reaction proceeds to completion, by treating all dissolved CO_2 as H_2CO_3. By 1914 Thiel and Strohecker had shown that only a small fraction, not yet accurately determined but now estimated at 1/400 – 1/1000, of dissolved CO_2 is hydrated to H_2CO_3 at equilibrium. As pointed out by Van Slyke (1921) the mass law equation

$$\frac{[\mathrm{CO_2}] \times [\mathrm{H_2O}]}{[\mathrm{H_2CO_3}]} = K \tag{38}$$

applies to reaction 37, and since $[H_2O]$ is constant the ratio $[CO_2]/[H_2CO_3]$ is constant at equilibrium. If the proportion of CO_2 hydrated to H_2CO_3 at equilibrium is designated by a factor then equation 34 becomes

$$\frac{[\mathrm{H^+}] \times [\mathrm{BHCO_3}]}{\mathrm{f}[\mathrm{CO_2}]} = K' \tag{39}$$

and

$$\frac{[H^+] \times [BHCO_3]}{[CO_2]} = fK' \tag{40}$$

Consequently the effect of assuming all dissolved CO_2 to be in the form of H_2CO_3 is to modify the true value of K' by an unknown factor f. Thus the convention, still in use, of representing all dissolved CO_2 as H_2CO_3 in the Henderson-Hasselbalch equation 35 introduces no error of calculation provided that the equation is applied only to equilibrium conditions.

Within the past ten years a new chapter has been added to the study of the acid-base balance in its relationship to the transport of CO_2 by the blood. The relationships which have been discussed above had up until 1928 been studied by methods which permitted equilibrium to be attained, without, as it now appears, sufficient regard for the shortness of time that the blood spends in the capillaries of the lungs and other tissues. Largely owing to the pioneer work of Henriques (1928) attention has been directed to the kinetics of the carbon dioxide processes in the blood. The results of this work have been extensively reviewed by Roughton and will be discussed here only in so far as they concern applications of the law of chemical equilibrium to the problem.

By reference to equations 2 and 4 above, expressions for the velocity of reaction 37 may be obtained, as follows. For the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ the reaction velocity

$$d[H_2CO_3]/dt = -k_{H_2CO_3}\,[H_2CO_3] \tag{41}$$

in which $k_{H_2CO_3}$ is the dehydration velocity constant of H_2CO_3, and for the reaction $CO_2 + H_2O \rightarrow H_2CO_3$

$$d[CO_2]/dt = -k_{CO_2}\,[CO_2] \tag{42}$$

in which k_{CO_2} is the hydration velocity constant of CO_2.

For the reversible reaction 37 there may be written

$$d[CO_2]/dt = -k_{CO_2}\,[CO_2] + k_{H_2CO_3}\,[H_2CO_3] \tag{43}$$

which, according to Roughton is well supported by experimental evidence. Study of the kinetics of this reaction by this derivation of the law of mass action led to the discovery of the enzyme, carbonic anhydrase, which greatly accelerates reaction 37 in both directions.

The establishment of a definite time factor in the hydration of CO_2 to H_2CO_3, and in the reverse process of dehydration of H_2CO_3 means

that the assumption of a constant $[CO_2]/[H_2CO_3]$ ratio, under all conditions, is no longer tenable. While, as previously stated, this involves no error of calculation in systems at equilibrium, the assumption of a constant ratio during the uptake of CO_2 in the tissues and its discharge in the lungs does introduce a possible error of thought with relation to the processes involved. For this reason we strongly support the plea of Stadie and O'Brien (1937) for the adoption of a system of notation which clearly indicates the processes and assumptions inherent in the application of the law of chemical equilibrium to the conditions in a $BHCO_3/CO_2$ system.

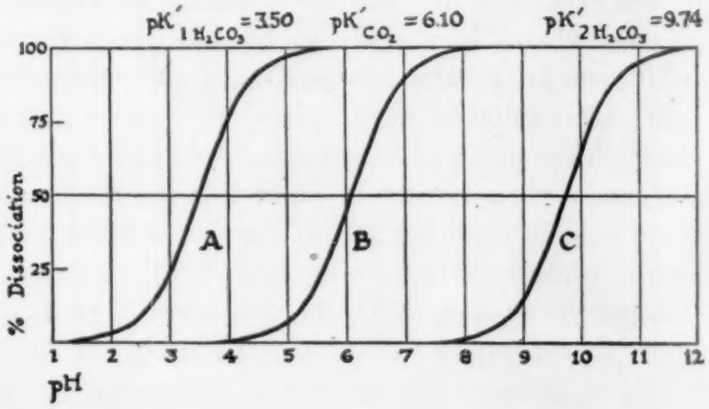


Fig. 1. "Dissociation curves" of carbonic acid. *% Dissociation* represents in Curve A — % of HCO_3^- + H_2CO_3 as HCO_3^- Curve B — % of HCO_3^- + CO_2 + H_2CO_3 as HCO_3^- Curve C — % of $CO_3^=$ + HCO_3^- as $CO_3^=$.

Stadie and O'Brien propose two equations for such a system, as follows:

$$\mathrm{pH} = \mathrm{p}K'_{CO_2} + \log \frac{[HCO_3^-]}{[CO_2]} \quad (\mathrm{p}K'_{CO_2} = 6.10) \tag{44}$$

$$\mathrm{pH} = \mathrm{p}K'_{1\,H_2CO_3} + \log \frac{[HCO_3^-]}{[H_2CO_3]} \quad (\mathrm{p}K'_{1\,H_2CO_3} = 3.5 \text{ approximately}) \tag{45}$$

The two equations give identical results when dissolved CO_2 and H_2CO_3 are at equilibrium, but only equation 45 expresses the condition of ionic equilibrium with non-equilibrium between CO_2 and H_2CO_3. These two equations are shown graphically in figure 1. The same figure includes a graphic representation of the equation

$$\mathrm{pH} = \mathrm{p}K'_{2\,H_2CO_3} + \log \frac{[CO_3^=]}{[HCO_3^-]} \quad (\mathrm{p}K'_{2\,H_2CO_3} = 9.74) \tag{46}$$

derived from equation 32.

Since curve B depends upon equilibrium between CO_2 and H_2CO_3, it has no quantitative meaning for the condition of non-equilibrium, while curves A and C depend only upon ionic equilibrium, assumed to be instantaneous. In considering conditions of non-equilibrium, curve B is of value not as representing the conditions at any moment but as representing the limiting conditions toward which the system tends to move. The pathways followed are *not* along curve B but along curve A, returning to curve B only at the instant equilibrium is attained.

For the contents of the red blood cells this limitation upon equation 44 probably introduces no serious error of thought. While hydration and dehydration of CO_2 in the red cells, during CO_2 uptake and discharge, occur only when CO_2 and H_2CO_3 are not in equilibrium, hydration and dehydration are greatly accelerated by the presence of carbonic anhydrase, and are probably very nearly as rapid as the interionic reactions. In both the lungs and the tissues, however, CO_2 must diffuse through the plasma with relatively little direct effect upon the H^+ concentration of this fluid, owing to the absence of carbonic anhydrase. The physiological implications of this are more fully discussed by Roughton but it is apparent that equation 44 and curve B of figure 1 give a somewhat incorrect conception of the conditions in the plasma during the brief periods of passage through the capillaries of the tissues and lungs.

Ionic activity. It will be noted that in equations 44 and 45 $BHCO_3$ has been replaced by the HCO_3^- of equation 31. This is in accord with modern theory, which assumes complete dissociation of $BHCO_3$ and replaces the concept of incomplete dissociation with that of activity.

In order to obtain equation 44 in the more generally applicable form of equation 31, Hastings and Sendroy (1925) made the assumption that the activity coefficient of dissolved CO_2 is unity, and since $a_{HCO_3^-} = f[HCO_3^-]$, wrote

$$pa_{H^+} = pK_{CO_2} + \log \frac{f[HCO_3^-]}{[CO_2]} \tag{47}$$

from which

$$pa_{H^+} = pK_{CO_2} + \log f_{HCO_3^-} + \log \frac{[HCO_3^-]}{[CO_2]} \tag{48}$$

They found a value of 6.33 for pK_{CO_2} at 38°C., and in accord with the Debye-Hückel theory a value of $-0.5\sqrt{\mu}$ for $\log f_{HCO_3^-}$. Consequently pK'_{CO_2} of equation 44 $= 6.33 - 0.5\sqrt{\mu}$, which becomes 6.10 at the

ionic strength of plasma. Use of the equation in this form is necessary when applied to solutions of ionic strength differing from that of plasma.

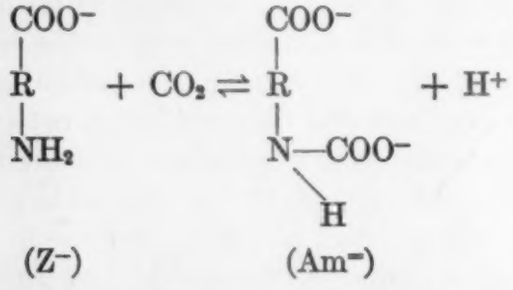
Buffer action. No review of the acid base balance would be complete without reference to the important concept of buffer action, which in theory and practice is a direct derivation of the law of chemical equilibrium as applied to the dissociation of weak acids and bases. Little can be added to the analysis of this subject in an important paper by Van Slyke (1922) except to point out the relationships of the buffer action of the system HCO_3^-/CO_2 to the considerations set forth in equations 44 and 45 above.

It is clear, from theoretical considerations alone, that such a system has in fact two buffer values. The first, defined by $pK'_{1_{H_2CO_3}}$ of equation 45, is the immediate buffer effect of the system HCO_3^-/H_2CO_3. The second, defined by pK'_{CO_2} from equation 44 is the buffer effect of the system HCO_3^-/CO_2 at equilibrium, and is the buffer effect which has been chiefly studied. The consequences of this double effect, depending upon the time required for equilibrium between dissolved CO_2 and H_2CO_3 to be attained have in part been discussed above. When a strong acid is added to the system HCO_3^-/CO_2 in the absence of carbonic anhydrase the immediate effect is a sharp increase in H^+ concentration to the pH as defined by equation 45, followed by a much slower rise in pH to that defined by equation 44. It was this phenomenon, first studied by colorimetric methods, which led Thiel and Strohecker to the conclusion that only a small fraction of dissolved CO_2 is hydrated to H_2CO_3 at equilibrium, and Faurholt (1924) to a study of the kinetics of the hydration process.

The rôle of hemoglobin in the acid-base balance. In the development of the modern theory of the acid-base balance the view was generally held up to 1920 that hemoglobin played a purely passive part, limited to its buffer action as a protein. It had previously been suggested, from several sources, that oxyhemoglobin is a stronger acid than reduced hemoglobin, but the matter was first studied systematically by L. J. Henderson (1920), who applied the law of chemical equilibrium to the problem and was able to make tentative estimates of the acid dissociation constants of the two forms of hemoglobin and to point out the far reaching physiological consequences of this unique property. For a review of this important application of the law of chemical equilibrium, which has been abundantly confirmed and extended, reference is made to Henderson's monograph (1928).

A further major discovery concerning the part played by hemoglobin

in the transport of CO_2 in the blood arose out of difficulties in applying equation 36 to solutions containing hemoglobin. Van Slyke, Hastings, Murray and Sendroy (1925) found that the presence of hemoglobin affected the values found for pK'_{CO_2}. Stadie and Hawes (1928) and Margaria and Green (1933) found further that the values for pK'_{CO_2} depended upon the state of the hemoglobin with respect to oxygenation. Henriques, and Margaria and Green suggested that these deviations could be most simply explained by the assumption that part of the total CO_2 was directly bound to hemoglobin as a carbamate. The history of the verification of this hypothesis, arising out of the application of the law of mass action, is extensively reviewed by Roughton. The carbamates represent a direct combination between amino acids or proteins and CO_2, as follows



(49)

Stadie and O'Brien (1935–36) have applied the law of mass action to reaction 49 in the case of simple amino acids. When no CO_2 is present as carbonate or bicarbonate they write

$$\frac{[Am^-] \times [H^+]}{[Z^-] \times [CO_2]} = K_{Am} \tag{50}$$

and find that this equation satisfactorily represents the experimental data under a variety of conditions. For their treatment of the total equilibrium of amino acid and CO_2, including both the carbamate and carbonate equilibria reference is made to their original paper.

Stadie and O'Brien (1937) have also applied the law of mass action to the carbamate equilibrium in the case of hemoglobin, and have accounted by this means for the distribution of CO_2 in its different forms in the blood. Their fundamental equations, slightly revised in agreement with Stadie (personal communication) are, for the non-carbonate equilibrium

$$Hb^- + CO_2 \rightleftharpoons HbAm^- + H^+ \tag{51}$$

$$\frac{[HbAm^-] \times [H^+]}{[CO_2] \times [Hb^-]} = K \tag{52}$$

from which, by combination with equation 44, the total equilibrium which includes bicarbonate as well as carbamate may be formulated.

Graphic representation of the acid-base balance. The acid-base balance is in its simplest form characterized by three variables from equation 36, viz., pH, total CO_2, and dissolved CO_2 (which includes H_2CO_3). From total CO_2 − dissolved CO_2 = HCO_3^- a fourth variable is obtained. Visualization of the relationships between these variables is greatly

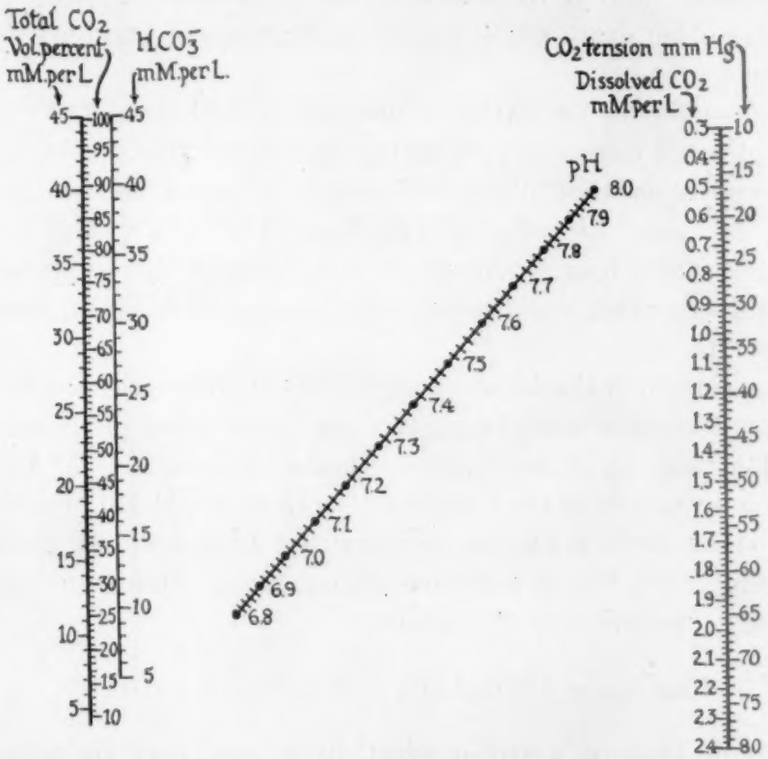


Fig. 2. Line chart for graphic calculation of serum or plasma values by the Henderson-Hasselbalch equation (equation 36 of text). A straight line through given points on any two scales cuts the other two scales at points indicating simultaneously occurring values.

facilitated by graphic analysis, of which Peters and Van Slyke give many examples. For use in calculation a previously unpublished line chart is here reproduced (fig. 2) which serves the same purposes as that published by Van Slyke and Sendroy (1928) but which has the advantage of theoretically exact scales for all of the variables.[8] Sendroy, Seelig, and Van Slyke (1934) have also published a theoretically exact

[8] The construction of line charts is greatly aided by the methods employed by Allcock and Jones (1932), involving the use of determinants.

line chart for calculation of any one of the three variables, total CO_2, dissolved CO_2, and pH from the other two in urine. By the use of appropriate values for the constants a similar chart could be constructed for the same three variables in plasma.

Equation 36 is peculiarly adapted to the use of triangular coördinates. Hastings and his co-workers (Hastings and Steinhaus, 1931; Shock and Hastings) have made use of this method of graphic analysis of the paths of displacement and of recovery of the acid-base balance. Sendroy (1938) gives other examples of recent applications of graphic calculation to this field.

THE DISSOCIATION OF CALCIUM CITRATE. Sabbatani (1901), to explain the effect of citrate in preventing the coagulation of blood, arrived at the hypothesis that calcium forms a complex ion with citrate. Hastings, McLean, Eichelberger, Hall and DaCosta (1934) confirmed the evidence which had accumulated in support of this hypothesis, and arrived at dissociation constants for the citrates of calcium, magnesium and strontium.

The dissociation of the alkaline earth salts of citric acid is complicated by the fact that this acid, being trivalent, has three dissociation constants. Hastings et al. were able to make observations of Ca^{++} concentrations by means of the frog heart method at pH 7.0 or above, in a range in which citric acid may be regarded as completely dissociated. In this range they found evidence that calcium citrate dissociates in two stages. The primary dissociation

$$Ca_3Cit_2 = Ca(CaCit)_2 \rightleftharpoons Ca^{++} + 2\ CaCit^- \tag{53}$$

appears to be that of a strong electrolyte, and may be regarded as complete.

The secondary dissociation,

$$CaCit^- \rightleftharpoons Ca^{++} + Cit^{=} \tag{54}$$

is that of a weak electrolyte, and Hastings et al. found it to be described by the mass law equation

$$\frac{[Ca^{++}] \times [Cit^{=}]}{[CaCit^-]} = K_{CaCit^-} = 10^{-3.22}, \tag{55}$$

at pH 7.4, 22°C. and $\mu = 0.155$ to 0.163.

Hastings et al. made further use of the law of mass action in arriving at this constant by a wholly chemical method. For a difficultly soluble

salt such as $CaCO_3$ in solution in equilibrium with its solid phase, the mass law equation may be written

$$\frac{[Ca^{++}] \times [CO_3^{=}]}{[CaCO_3]} = K \tag{56}$$

and since under these conditions $[CaCO_3]$ in solution may be assumed to be constant

$$[Ca^{++}] \times [CO_3^{=}] = K_{s.p.CaCO_3} \tag{57}$$

which represents the familiar solubility product constant.[9] By equilibrating citrate-containing solutions with solid $CaCO_3$ at known CO_2 tensions the Ca^{++} concentration was calculated, and by this means a value of $10^{-3.31}$ for K_{CaCit^-} was arrived at.

Muus and Lebel (1936) by calculation of Ca^{++} concentrations from the solubility product constant of calcium iodate, by the method just described, found a value of $10^{-3.21}$ for K_{CaCit^-} thus confirming the value found by the frog-heart method. These workers were also able to extend their observations to a lower pH range in which the dissociation

$$HCit^{=} \rightleftharpoons H^{+} + Cit^{\equiv} \tag{58}$$

is incomplete and may be represented by the mass law equation

$$\frac{[H^{+}] \times [Cit^{\equiv}]}{[HCit^{=}]} = K_{3\,H_3Cit} \tag{59}$$

In this range they postulated the reaction

$$CaHCit \rightleftharpoons Ca^{++} + HCit^{=} \tag{60}$$

and found that

$$\frac{[Ca^{++}] \times [HCit^{=}]}{[CaHCit]} = K_{CaHCit} = 10^{-2.3} \tag{61}$$

From equations 55, 59, and 61 we have constructed a grid nomogram (fig. 3) with four variables, viz., Total Ca, Total Citrate, pH, and Ca^{++}, which facilitates the calculation of Ca^{++} concentrations at varying pH, and has been made use of by Ransmeier and McLean (1938) for the calculation of Ca^{++} concentrations in citrated plasma.

[9] The applications of the law of chemical equilibrium to the solubility of difficultly soluble salts are not here reviewed. The present status of the problem of the solubility relationships of the salts of bone, which is beset by a number of difficulties, is reviewed by Logan and Taylor (1937).

The dissociation constant of magnesium citrate has been found (Hastings et al., 1934) to be approximately that of calcium citrate.

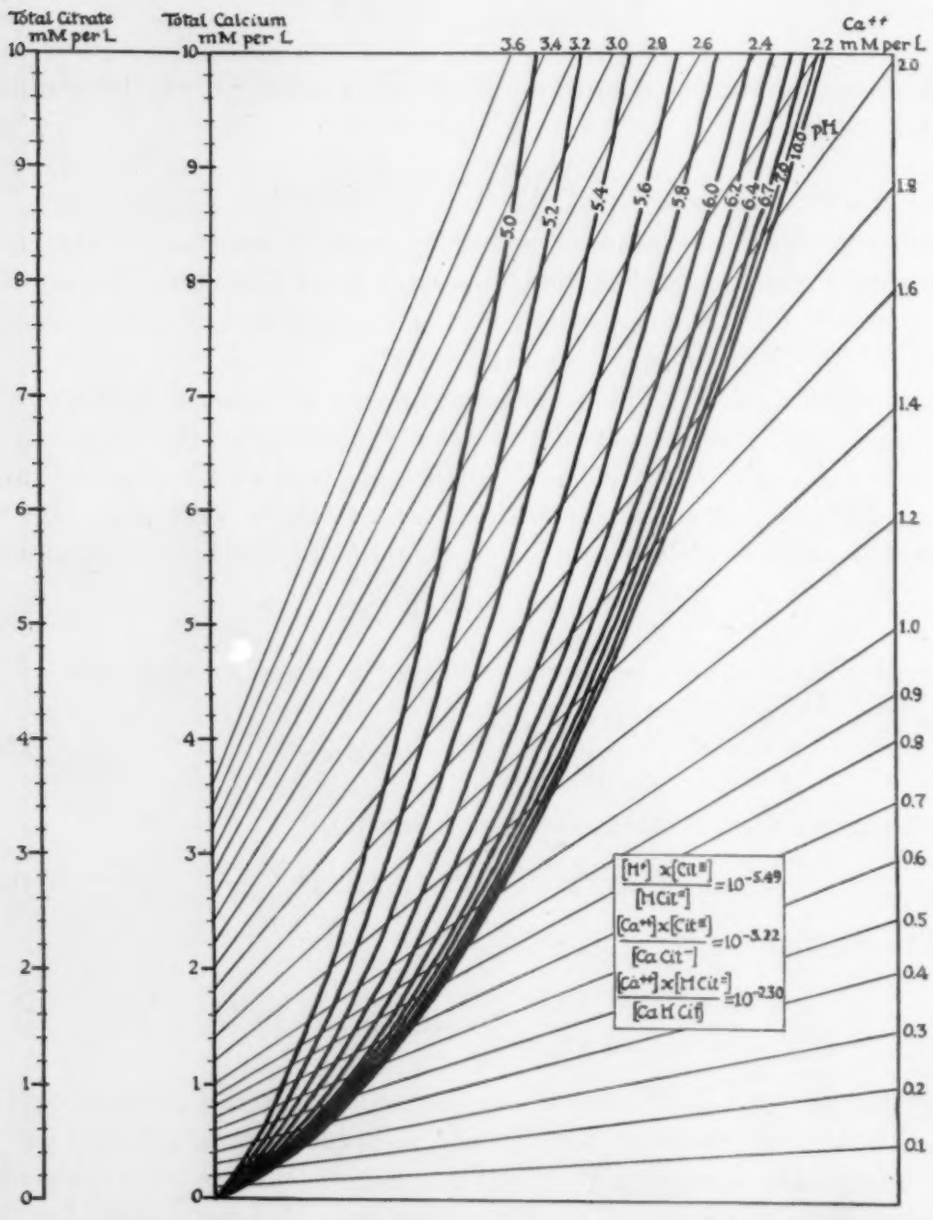


Fig. 3. Grid nomogram for calculation of Ca^{++} concentrations from Total Ca, Total Citrate, and pH. If a straight line is drawn through given points on Total Ca and Total Citrate scales, the Ca^{++} concentration can be read by interpolation between iso-Ca^{++} lines at the point where the line drawn intersects the line representing the given pH. I am indebted to Solomon J. Klapman for construction of this nomogram.

When calcium and magnesium are both present in a citrate-containing solution each influences the ionization of the other. A grid nomogram with four variables, viz., Total Ca, Total Mg, Total Citrate, and Ca^{++}, similar to figure 3, may be readily constructed for use at pH above 7.0, in the range in which citric acid is practically completely dissociated.

THE STATE OF CALCIUM IN PROTEIN-CONTAINING FLUIDS. Using the frog-heart method for estimation of Ca^{++} concentrations, McLean and Hastings (1935) were able to show that calcium proteinate dissociates as a weak electrolyte, and that the ionization of calcium in a protein-containing fluid, such as serum or plasma, may as a first approximation be described by the equation

$$CaProt \rightleftharpoons Ca^{++} + Prot^{=} \tag{62}$$

and the corresponding mass law equation

$$\frac{[Ca^{++}] \times [Prot^{=}]}{[CaProt]} = K_{CaProt} \tag{63}$$

the value found for K_{CaProt} in the case of the combined serum proteins being $10^{-2.22}$.

An uncertain factor in this equation is the expression of protein in terms of molal concentrations with respect to its calcium-combining groups. McLean and Hastings assumed that the figures obtained from titration curves of protein with base were applicable, at the same time pointing out the weakness in this assumption.

A second uncertain factor is the assumption that protein behaves toward calcium as if the former were a series of divalent ions. It now appears that this assumption is not necessary, and that the equations may equally well be written

$$CaProt^{+} \rightleftharpoons Ca^{++} + Prot^{-} \tag{64}$$

$$\frac{[Ca^{++}] \times [Prot^{-}]}{[CaProt^{+}]} = K_{CaProt^{+}}, \tag{65}$$

since the experimental data show merely that there is a one to one relationship between calcium and protein ions, and are not sufficiently accurate to distinguish between equations 63 and 65.

Greenberg, Larson and Tufts (1934–35) by an ingenious handling of the mass law equation, have avoided both of these uncertainties. They write the equation,

$$\frac{\text{Total Protein}}{[CaProt]} = \frac{1}{A} + \frac{B}{[Ca^{++}]} \tag{66}$$

in which Total Protein is expressed in grams per liter, and [Ca^{++}] and [CaProt] in molal concentrations. A then becomes the factor necessary to convert Total Protein to terms of molal concentrations, and $A \times B$ becomes the equilibrium constant K. When observed data obey the mass law a straight line is obtained on plotting $\frac{\text{Total Protein}}{[\text{CaProt}]}$ against $\frac{1}{[Ca^{++}]}$, the value A is given by the reciprocal of the point of intersection of the straight line on the Total Protein/[CaProt] axis, and the value of B is given by the slope of the line.

By this method Greenberg, Larson and Tufts found the calcium-combining power of protein at $Ca^{++} = \infty$ to be much lower than that calculated from the titration curves with base and found a value of $10^{-2.75}$ for K_{CaProt} for serum proteins as compared with $10^{-2.22}$ as reported by McLean and Hastings, the difference in the numerical value for K_{CaProt} being in part attributable to the lower protein factor. Larson (1937) has extended these observations and now finds a somewhat higher protein factor and a value of $10^{-2.44}$ for K_{CaProt}.

These discrepancies are no doubt due in part to the differences in method for estimation of Ca^{++}. The method of Greenberg, Larson and Tufts for estimation of the protein factor, while sound in principle, depends upon extrapolation from the conditions at low Ca^{++} concentrations to those at $Ca^{++} = \infty$. For Ca^{++} concentrations within the range found in the serum of man the differences calculated by using different protein factors are generally within the limits of observational error. Further refinement of the values for the protein factor and K_{CaProt} will depend upon methods for determining Ca^{++} concentrations with greater accuracy and over a wider range.

The calculation most commonly made by the use of equation 63 is that of Ca^{++} from total calcium and total protein. Starting with the equations

$$\text{Total Ca} = Ca^{++} + \text{CaProt} \tag{67}$$

$$\text{Total Protein} = \text{Prot}^{=} + \text{CaProt} \tag{68}$$

and the mass action equation 61, there may be derived the equation

$$[\text{Total Ca}] = \frac{[Ca^{++}] \times [\text{Total Prot}]}{[Ca^{++}] + K} + [Ca^{++}] \tag{69}$$

from which the cartesian nomogram published by McLean and Hastings is readily constructed. McLean (1935) has published a simplified method for the construction of this and of similar nomograms.

Previous writers had shown that the level of total calcium in the serum, in the absence of any disturbance in calcium metabolism, is directly correlated with the concentration of total protein, and had expressed this relationship in the form of the empirical regression equation

$$\text{Total Ca} = m\ \text{Total Protein} + b \tag{70}$$

McLean and Hastings noted the similarity of this equation to equation 69 above, assuming the Ca^{++} concentration in the fluids studied to be approximately constant. From the data in the literature they were able to calculate values for K_{CaProt} in fair agreement with those arrived at by the frog-heart method.

OXIDATION-REDUCTION EQUILIBRIA. The modern use of oxidation-reduction potentials in the study of biological oxidations is an outgrowth of the pioneer work of Clark (1923), who applied the law of chemical equilibrium to oxidation-reduction systems, and showed how the electrode potential of such a system depends upon the equilibrium constant. For general treatment of the subject reference is made to the reviews by Clark (1934, 1938).

Oxidation and reduction are characterized by the transfer of electrons. The essential feature of oxidation is the loss of electrons, which may be accompanied by the addition of oxygen or the loss of hydrogen. Reduction is the reverse of oxidation. For our present purposes it is convenient to consider a case in which the reaction involves only the transfer of electrons.

$$H_2 + 2\ Fe^{+++} \rightleftharpoons 2H^+ + 2\ Fe^{++} \tag{71}$$

At equilibrium we may write

$$\frac{[H^+]^2 + [Fe^{++}]^2}{[H_2] + [Fe^{+++}]^2} = K^{10} \tag{72}$$

Since this reaction involves only the transfer of electrons we may let it occur in two half-cells, with provision for the passage of electrons.

$$H_2 \rightleftharpoons 2H^+ + 2e \tag{73}$$

$$e + Fe^{+++} \rightleftharpoons Fe^{++} \tag{74}$$

If we now fix the first half-cell at the standard condition of 1 atmos-

[10] To be theoretically exact this subject should be developed in terms of activities. For the sake of simplicity, activity coefficients are here neglected.

phere of H_2 and N/1 H^+ the ratio $[H^+]^2/[H_2]$ becomes unity, and when the second half-cell is in equilibrium with the first, equation 72 becomes

$$\frac{[Fe^{++}]}{[Fe^{+++}]} = \sqrt{K} \tag{75}$$

It is seen that under these standard conditions, and at equilibrium, the proportion of Fe^{++} to Fe^{+++} is fixed by the equilibrium constant. If on the other hand we fix the ratio Fe^{++}/Fe^{+++} at unity and permit equilibrium to be attained by a change in the other half-cell the ratio $[H^+]^2/[H_2]$ is determined by the equilibrium constant. In both cases the tendency of reactions 73 and 74 to proceed to the right or the left, or in other words the tendency of any system to give up or accept electrons, is described by the value of K. Thus if we allow K in equation 75, and hence in equation 72, to decrease, reaction 74 proceeds to the left, with loss of electrons or oxidation of Fe^{++} to Fe^{+++}. If two oxidation-reduction systems are mixed, and if a reaction occurs, the system with the lower value for K will lose electrons, or become oxidized, while the system with the higher value for K will accept these electrons and become reduced. The prediction of the possibility and of the direction of any oxidation-reduction reaction thus requires nothing more than a knowledge of the value of the equilibrium constant for each of the systems. This was clearly pointed out by Clark (1923) and the subject has recently been reviewed in detail from this standpoint by Hogness and Johnson (1937), without reference to thermodynamics.

Since the characteristic method of study of oxidation-reduction systems is by measurement of the electrical potentials of such systems, and since the chief concern in the investigation of biological oxidations is with changes in free energy, thermodynamic treatment of this subject is at once more useful and more descriptive.

Substituting $-\Delta F = nEF$, in which n is the number of equivalents reacting, E the electromotive force, and F the faraday, in equation 22 above, and writing in terms of concentrations we have

$$E = \frac{RT \ln K}{nF} + \frac{RT}{nF} \ln \frac{[A]^a \times [B]^b}{[C]^c \times [D]^d} \tag{76}$$

or for the specific case of the reaction shown in equations 71 and 72

$$E = \frac{RT \ln K}{2F} + \frac{RT}{2F} \ln \frac{[H_2] \times [Fe^{+++}]^2}{[H^+]^2 \times [Fe^{++}]^2} \tag{77}$$

from which

$$E = \frac{RT\ln K}{2F} + \frac{RT}{F} \ln \frac{[H_2]^{\frac{1}{2}} \times [Fe^{+++}]}{[H^+] \times [Fe^{++}]} \tag{78}$$

By reference to the standard hydrogen electrode (N/1 H^+ and one atmosphere H_2) $[H_2]^{1/2}/[H^+]$ becomes unity, and if Fe^{+++}/Fe^{++} is unity we have, since $\ln 1 = 0$

$$E_h = E_0 = \frac{RT\ln K}{nF} \tag{79}$$

in which E_h is the electrode potential referred to the standard hydrogen electrode and E_o is the potential under the same standard conditions of reference, and at half oxidation of the oxidation-reduction system. E_o thus characterizes the system, and since E_o is a logarithmic function of the equilibrium constant the system may equally well be characterized by K, as shown above.

For conditions other than half oxidation, but at the standard conditions for reference, equations 78 and 79 become

$$E_h = E_0 + \frac{RT}{F} \ln \frac{[Fe^{+++}]}{[Fe^{++}]} \tag{80}$$

or more generally, for any system involving n equivalents

$$E_h = E_0 + \frac{RT}{nF} \ln \frac{[\text{Oxidant}]}{[\text{Reductant}]} \tag{81}$$

which is the standard equation for oxidation-reduction potentials.

THE CHANGE IN FREE ENERGY. From the above, and from equation 23 it may be seen that the free energy change, ΔF, of a reaction, the equilibrium constant, and the electromotive force of a system, all under standard conditions, are related as follows:

$$-\Delta F = RT\ln K = nEF \tag{82}$$

and it has been pointed out in the introduction that the data obtained by a variety of methods may be expressed in the terms most appropriate to the subject under consideration.

The free energy change of a reaction may be determined by purely thermal methods, but until recently applications of such methods to reactions of biological interest have been few. Borsook (1935) has undertaken the systematic collection of thermochemical data and has extensively reviewed the possibilities of their application. Brief refer-

ence will be made here to a specific case illustrating the relationships expressed in equation 82.

In the presence of an appropriate enzyme succinic acid is oxidized to fumaric acid. The system is of itself not electromotively active, but if a small amount of a dye, such as methylene blue, is added the potential of the system is transferred by the dye to the electrode. Moreover, if the constants for the dye system are known the potential of the system with which it is in equilibrium, and consequently its equilibrium constant, may be derived from colorimeter readings. For the succinate-fumarate system the equilibrium constant was determined colorimetrically by Quastel and Whetham (1924), colorimetrically and electrometrically by Thunberg (1925), electrometrically by Lehmann (1930), and by Borsook and Schott (1931).

Borsook and Schott also calculated the free energy change for the same reaction from thermal data, the details of their calculations being given also by Borsook (1935). They compared the values for ΔF arrived at by this method with those derived from the data obtained by other methods, and found a remarkable agreement. On account of certain advantages of the thermal method, as stated by Borsook, it seems probable that the thermal method for studying free energy changes may come into more general use.

A thermodynamic principle, as yet applied to but few reactions of biological interest, is that the free energy change is a quantity determined by the initial and final states of the system. For a catenary reaction, of which there are many examples in metabolic processes, the change in free energy may be calculated from the initial and final states, without reference to the intermediate reactions. Thus Burk (1929) has calculated the free energy of the breakdown of glycogen to lactic acid in muscle from thermal data. Moreover, if the data for the intermediate reactions are available the change in free energy from the initial to the final states may be calculated by combining these data. Some examples are given by Wilson and Peterson (1931).

Earlier applications of thermochemistry to reactions of biological interest were chiefly concerned with the heat of reaction, a quantity which is of itself of less significance than the change in free energy. The heat of reaction of hemoglobin with oxygen was studied by Brown and Hill (1923) and their paper illustrates the applicability of such data to the study of chemical equilibrium.

ENZYMATIC REACTIONS. The study of enzymes, and of the reactions of biological interest catalyzed by them, has been in large part de-

pendent upon the application of the law of chemical equilibrium to oxidation-reduction potentials and more recently upon the use of thermal data for calculation of free energy changes. Study of the kinetics of enzymatic reactions has also been greatly aided by derivations from the law of mass action. No attempt is made here to review the voluminous literature of this special field. For consideration of these important applications of the law of mass action the forthcoming review by Barron may be consulted.

CONCLUSION

So far as the author is aware this is the first attempt to review the contributions of the law of chemical equilibrium to the study of biological problems. It might be thought *a priori* that this law, being a statement of the conditions in a chemical system at equilibrium, might be of very limited application to biological processes. To the contrary, its rôle in the elucidation of the mechanisms concerned with the acid-base balance and in the discovery of physiologically important properties of hemoglobin can hardly be overestimated, and it has opened the way for equally significant progress in the understanding of the complexities of intermediary metabolism. It should be increasingly useful as biology becomes more and more concerned with the analysis of chemical processes in the living organism.

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PLANT GROWTH HORMONES

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I. METHODS. Of the several lines of approach which have elucidated the rôle of hormones in plant growth, that depending on the elongation of seedlings has been the most important. Since such measurements have been widely used as standard assay methods, these methods will first be outlined.

The historical development of the subject has recently been discussed in two monographs (32, 186) and may therefore be omitted. The concept that growth by elongation is regulated by a hormone is based on the observation that in the coleoptile of the oat seedling (*Avena sativa*), grown in darkness, growth is largely controlled by the extreme tip. If this tip is removed the growth rate is greatly reduced; if it is replaced symmetrically the growth is accelerated, and if it is replaced asymmetrically only the side to which it is applied is accelerated, so that the coleoptile curves. Went (182) showed that if the tip be placed on agar, the agar acquires the same properties of growth promotion, which are therefore due to the diffusate. Growth-promoting activity in any preparation may be detected by applying it (usually dissolved in agar) to one side of a decapitated coleoptile. Under certain conditions the curvature so obtained is proportional, within limits, to the concentration of the active substance. With different modifications this so-called *Avena* test has been widely used for 10 years.

The minimum concentration of the active substance giving curvature, the maximum curvature obtainable, and the type of relationship between concentration and curvature vary with the conditions of the test. These include *a*, the medium in which the plants are grown, which may be water, sand, soil or sawdust (182, 32); *b*, the age of the plants, about 74 hours' growth at 24–25° being optimal; *c*, the time between decapitating and putting on the agar block (186, 141); *d*, the concentration of agar used and the method of mixing the test solution with the agar (169); *e*, presence or absence of the seed, removal of the seed causing the curvature to continue to increase for a longer time (145); *f*, the light

in which the plants are grown. Usually red or orange light is used, but the tests may also be carried out in diffuse daylight (155, 156). Some action of the light, particularly if yellow is used, is apparently responsible for variations in sensitivity of the plants at different hours of the day (92). The test is largely independent of toxic metallic cations or other inactive substances, and it is also, despite claims to the contrary (8), independent of pH unless the preparations are strongly buffered (13, 169).

Curvature methods have also been used on other seedlings, notably *Raphanus* (124) and *Cephalaria* (155, 156). The latter are apparently more sensitive than *Avena* to the lowest concentrations of growth hormone.

Activity may also be measured by straight growth. This can be done by applying the agar symmetrically to the cut surface (168), by placing an isolated coleoptile upright with one end dipping in the solution (82), or by immersing short sections of coleoptile in shallow layers of the test solution (15, 140). Unlike curvature, straight growth is usually measured after a period of 6 hours or more. The elongation is is more nearly proportional to the logarithm of the concentration of hormone than to the concentration itself. Measurements on immersed sections are affected by some of the factors mentioned above, and also, very markedly, by the presence of sugar or neutral salts (140, 169). Straight growth measurements have also been employed on stems of pea, and hypocotyls of lupin, sunflower and radish.

Another method depends on the fact that when elongating organs are slit longitudinally and immersed in active solutions, the two slit halves curve inward (183). The inward curvature is roughly proportional to the logarithm of the hormone concentration. This has been used with stems of etiolated pea seedlings (183, 186, 130, 170), oat coleoptiles (170) and dandelion flower-stalks (82, 170).

The results obtained by these three types of assay, the "Avena test", straight growth measurements and the slit stem test, show important differences (see section VII). The first is the most specific, some substances which show activity by the other methods, or in related experiments, being inactive in the *Avena* test (163, 70).

II. The auxins and their action. The substances active in the above tests have been termed auxins. In view of their many physiological activities it will not be possible to consider all the fields which have developed, but emphasis will be placed on the most active lines of work.

In the last few years it has become clear that the growth hormones of this type control numerous processes in the plant besides simple elongation. In roots they promote elongation only slightly and only at excessively low concentrations (4, 60, 64, 58, 162), while higher concentrations strongly inhibit root elongation (see 186). The development of buds is also inhibited (167, 117, 54, 151) and to this the so-called "apical dominance" is due.

The initiation of roots at the base of cuttings is promoted, and largely controlled, by hormones of this group (see p. 535), and this is leading to commercial use of these substances, either pure or in the form of patent preparations (some of them greatly overpriced) by horticulturists and propagators. The formation of roots by the young seedling may also be increased (108, 171). Lastly, the substances activate cell division in the cambium and cause swellings in both shoot and root tissue (see section V).

Chemically the auxins are a rather loosely-related group. Since the isolation of the first of them by Kögl and Haagen-Smit (86) and the later identification of indole-3-acetic acid as a highly active substance for all the above functions (90, 94, 166, 161) a large number of substances have been found to have activity. All are ether-soluble unsaturated organic acids or their salts or esters. The substance first isolated from urine and from corn-germ oil was later (89) shown to be a cyclopentene derivative of trihydroxy-valeric acid, and was named auxin *a* or auxentriolic acid. The corresponding ketohydroxy-acid (auxin *b* or auxenolonic acid) was also obtained (88). The chemistry of the auxins will be discussed in section VII, and that of other plant growth substances in section VI.

The amounts of the auxins causing growth responses are extremely low. About 10^{-8} mgm. suffice to cause 1° curvature in the *Avena* test, while a 10^{-10} molal solution will produce detectable increase in the growth rate of corn roots.

III. THE FORMATION OF AUXINS IN THE PLANT. The essential feature of the control of growth by a hormone is the production of the hormone in minute amounts in special centers and its transport through the tissues. In plants, without a generalised circulation, the transport of organic substances is still not clearly understood, and the transport of auxin is a special case, because it is typically a polar movement, i.e., the auxins move, in the shoot, from the apex towards the base. This polar direction of transport holds also for externally applied auxins (with certain exceptions), providing their concentrations are comparable

with those existing in the normal plant. Concentrations 100 to 1000 times the physiological levels apparently overcome polarity of transport. On account of this general unidirectional movement, the points at which auxin is produced are of considerable importance.

Formation in the seed. Auxin is present in many seeds, but in cereals its amount usually falls off rapidly during germination and the first days of growth. However, in the oat it is not present in the dry seed (46) but appears in the endosperm as soon as the seed is wetted, being presumably set free from some inactive "precursor". Since the auxin activity of some seed oils is increased by short hydrolysis with acid or lipase (88) it has been pointed out (186) that the precursor may be an ester of auxin *a* or *b*, these esters being themselves inactive (87). The physiology of tryptamine, which appears to be converted to indoleacetic acid in the plant (145) and the similar behavior of tryptophane with some fungi (161) suggests, however, that the precursor might be an amino-compound.

Cholodny (46) considered that the auxin in the seed plays the part of a germination hormone, i.e., it stimulates the initiation of seedling growth. This, however, is made less probable by the fact that application of indole-acetic acid to isolated oat embryos does not promote the outgrowth of the young roots, but only inhibits them (162). It is also not supported by the behavior of apple seeds, which do not germinate (under the experimental conditions used) unless freed from the endosperm (177), although both seed and endosperm contain much auxin.

On the other hand three pieces of evidence indicate that the auxin of seeds plays an important part in seedling growth, though perhaps not in the actual germination process. The first of these is the rapid disappearance of auxin during seedling development (91, 46). The second is the observation that pine seeds, which do not germinate when freshly picked, do so after storage for some months (116). Such seeds contain little auxin at first but the amount increases during low temperature storage to a high value at the time when they are able to germinate. The third is the recent finding (47, 171) that treatment of oat seeds for a short time with auxin greatly increases their subsequent growth. At first the roots are inhibited in elongation but after the treatment they are accelerated and also increased in number (108, 171); there is a general increase in vegetative growth and even some hastening of flowering, while the percentage of seeds which germinate may also be increased. Certain other seeds show effects of the same sort (6). There are similarities between these effects and those resulting from

vernalization, i.e., low temperature storage of seed in the moist state. The effects of vernalization on vegetative growth, but not those on flowering, may be due to the exposure of the embryo to the auxin formed within the seed during the storage period.

Formation in the oat coleoptile. Since the coleoptile has been so extensively studied, much of the first work on auxin formation relates to this object. Here formation is limited to the tip, but when the tip has been removed a new zone of auxin formation forms at the apex of the stump after two to three hours. This "regenerated physiological tip" shows no histological differentiation, so that auxin formation is not necessarily associated with special cells.

The isolated tip after removal excretes auxin into agar at an approximately constant rate for 2 hours, then at a gradually decreasing rate for several hours more (182, 180); it must therefore contain stored precursor. The amount of auxin formed increases during the growth of the coleoptile, roughly parallel to the increase in growth rate for the first 70 hours (186).

It has been claimed by Pohl (131; see also 127) that the auxin in the tip is not formed there but comes up from the seed. This, however, is improbable on account of the polar transport in the coleoptile, which prevents auxin from moving to the tip. If agar blocks are placed on the stump of the seedling after removal of the coleoptile, no true auxin enters them, but when the blocks are subsequently applied to standard test coleoptiles, they produce a slow and much-delayed curvature (145). This is ascribed to accumulation of precursor in the block, and its slow conversion to auxin by the test plant. Hence it is probably the precursor which travels up from the seed to the tip and is there converted to the active form. The scutellum, which is unlike the endosperm in that it forms no auxin when wetted, nevertheless contains a store of precursor (178). In view of the rapid production of auxin on wetting the seed (46) there may be two kinds of precursor, one—in the endosperm—readily converted into auxin, the other—in the scutellum—not converted *in situ*, but transported to the coleoptile tip and converted there.

The regeneration of auxin after removal of the tip is to be ascribed to new accumulation of precursor at the most distal remaining part. If the store of precursor is removed by removing the seed, this regeneration in the apex of the stump does not occur (145). It is worth noting that all this auxin formation goes on in darkness, the seed being provided with enough auxin (in precursor form) for its growth until the plant reaches the light and photosynthesis can begin.

Formation in buds. If dormant buds of Windsor bean (*Vicia Faba*) are cut off and placed on agar, almost no auxin diffuses out from them, but if growing buds are treated in the same way they yield relatively large amounts (167). In trees, the amount diffusing from a bud in spring is correlated with the amount of growth of the stem below, while the time at which auxin formation begins agrees with the time of inception of growth (153, 199, 11, 128).

This production of auxin is presumably not a photosynthetic process, but, as in the coleoptile, is from precursor stored in the bud. It is greatly reduced by nitrogen starvation (9, 67), but since nitrogen starvation affects many processes this may not be a direct effect. It is also reduced by zinc deficiency, the first detectable symptom of which is a reduction in the auxin diffusing out of the buds (148). Since the need of plants for zinc is extremely small, this may throw important light on the nutritive rôle of zinc. The effect may be a truly decreased formation, or an increased destruction, of the auxin.

Formation in leaves. The time of most active auxin formation in leaves appears to be when they are young (167, 7, 65). This may be a continuation of the auxin-forming process which goes on while the leaves are still in the growing bud. As the leaves develop, however, auxin formation becomes a photosynthetic process (since it is dependent on illumination of the leaves (7)). It is possible that the precursor is formed only in light, mainly in older leaves, and is then transported to buds and stored there. The subsequent conversion to auxin would then take place independently of light, as in etiolated seedlings. There is evidence that another photochemical reaction in the leaf produces substances which promote flowering. Thus some plants initiate flower primordia only if exposed to "short days", while other plants require longer day lengths (14 hours or more). This influence of length of day has to be exerted on the leaves and not on the buds themselves (40). Nevertheless the production of auxin in leaves is, in all the cases studied, greater when the leaves are kept in long days than in short (41). This indicates that auxin bears no direct relation to the process of flower initiation. On the other hand, in one plant at least (*Circaea*) a bud which would normally become a flower may be converted to the vegetative condition by auxin application (55), so that the amount of auxin which reaches a bud must exert some action on flower formation. To what extent a special hormone is involved in flower initiation cannot yet be concluded.

Formation in roots. There has been some uncertainty as to whether auxin is actually formed in roots or not. Auxin may be obtained from

root tips by diffusion into agar (75), or better into agar containing 5–10 per cent dextrose (33, 118). In such sugar-fed roots van Raalte (133) showed that the total amount of auxin may apparently increase for a time, i.e., new formation is occurring. Auxin may also be obtained by direct extraction of either root tips or root bases with chloroform or ether, the solvent being then evaporated and the auxin taken up in water and tested on *Avena* (160). Both by extraction (160, 34, 133) and by diffusion (33) the amount present is found to be greatest near the tip. This may indicate actual production in the tip, but could also be due to accumulation in the tip either of auxin or of precursor derived from the seed or shoot. Auxin can be transported in both directions in the root (66), but more vigorously away from the tip than towards it (45, 118) which makes its accumulation at the tip less likely. Since the root is parasitic upon the shoot both for its carbohydrate supply and also for its vitamin B_1, it would be of interest if it were independent of the shoot for its auxin supply.

It might be expected that the behavior of isolated root-tips growing in culture media would elucidate this problem. With such root-tips, using peas and corn, Fiedler (60) found that although auxin is present at the time of cutting off, it disappears very rapidly from the isolated root, apparently by oxidative destruction. This would suggest that auxin is not being formed under these conditions although the roots are growing. On the other hand Nagao (119) found that in sunflower roots, after 6 days in culture, about the same amount of auxin was present per root as at first. Also the findings of Fiedler have been disputed by van Overbeek and Bonner (129), who, using pea roots grown under more favorable conditions than those of Fiedler, found abundant auxin present in the isolated root, even over periods as long as three weeks. The negative results of Fiedler are ascribed either to his cultural conditions or to the extraction technique used (160), which has since been considerably improved upon (127, 56). Auxin is thus present not only in normal roots but also in isolated roots grown on a purely synthetic medium, though its production in such cultured roots is still not established.

IV. Auxin inactivation. It has been shown by Thimann (160) that auxin is readily inactivated by extracts of leaves or other tissues and that this inactivation is particularly rapid if the plant studied possesses the peroxidase-catechol-oxidase enzyme system. Auxins are known to be readily oxidized *in vitro*, and there is no doubt but that the inactivation in plants is an enzymatic oxidation. Auxin is probably

inactivated in an analogous manner in growth; it has been shown, for example, that when auxin is applied to the oat coleoptile, only a small portion is recoverable (28). Excessive auxin destruction of this type may result in dwarf stature of the adult plant, as has been shown by van Overbeek (126) in the dwarf "nana" form of corn. The "nana" gene (a simple Mendelian recessive) causes excessive peroxidase activity, which is associated with abnormally great auxin destruction, causing dwarf growth. Excessive auxin destruction may also result in failure of the normal inhibition of buds (see section V) and in consequent "bushy" growth habit of the plant. X-radiation inactivates indole-acetic acid solution *in vitro*, the mechanism in this case also being an oxidative one (144). The inhibitory effects of x-radiation upon plant growth are thus in large part due to a similar auxin destruction within the tissues (144).

Ultra-violet light inactivates auxin *a* (96, 98), the reaction here being not an oxidation but a molecular rearrangement, in which the double bond migrates from the ring to the sidechain. The inhibitory effects of ultra-violet on plant growth (reviewed in 132) may well be due in part to this same reaction. Indole-propionic acid (72) and other indole derivatives are also inactivated by ultra-violet. Even visible light can inactivate auxins in presence of a sensitizing pigment. Thus, indole-acetic acid solutions are rapidly inactivated by light in presence of eosin (144), while auxin *a* is inactivated *in vivo* by visible light, the reaction being perhaps sensitized by carotenoid pigments (99).

V. The effects of auxin on growth. The use of the oat coleoptile in much of the auxin work was discussed in section 1. In this organ growth is dependent on the presence of auxin, and there is excellent evidence (182, 163, 140) that without auxin no growth can occur. Similar relations apparently hold for hypocotyls and stems of all plants investigated. There is no species-specificity known among the auxins, and the elongation of all above-ground parts of the higher plants probably takes place under the control of the natural auxin formed in them or transported to them.[1] That so many objects have been used for the bio-assay of auxin, and that many more show qualitative growth responses, either from their native auxin source or from artificially-applied auxins, testifies to the general nature of the growth-promoting effect.

[1] With the exception of leaf-blades, whose auxin relations are not clear. Isolated leaf-blade tissue does not increase in area in auxin solutions. Auxin may be needed for their growth but it is doubtless formed in sufficient amount *in situ* and is not the controlling factor.

Asymmetrical growth: tropisms of shoots. One of the best examples of the controlling effect of auxin on growth is afforded by the tropisms. In these, according to the view put forward independently by Cholodny (43) and Went (182), curvature is due to unequal distribution of auxin between the two sides of the organ. In geotropism, i.e., the bending of shoots away from gravity, Dolk's researches (52) established that the growth of the lower side of the coleoptile is accelerated, that of the upper side decreased. This is the result of a redistribution of auxin between the two sides, about 63 per cent being found on the lower side. In stems and hypocotyls similar values have been obtained (see 186, p. 165).

In positive phototropism, i.e., the bending of shoots towards light, there is a similar auxin redistribution, more being found on the shaded side (182, 124). With greater quantities of light, i.e., higher values of intensity $\times$ time, the curvature is reversed, the coleoptiles bending away from the light, and here, as expected, the greater quantity of auxin is found on the illuminated half (186). Phototropism, however, involves more than simple auxin redistribution. There is also the "light-growth reaction," studied by Blaauw, which is essentially a decrease in the effectiveness of auxin in light (124). This is probably due to the photoinactivation discussed in section 4.

The way in which light and gravity cause this asymmetric distribution of auxin is unknown. However, it must be remembered that the normal, unstimulated, transport of auxin is also an unknown process, so that the one can probably not be understood without the other.

Asymmetrical growth also occurs apart from tropisms. In *Tradescantia*, while in the vertical position, the lateral branches curve toward the main stem (epinasty), due to an auxin redistribution (176). Here the direction of curvature is determined by the morphology of the plant, although the stimulus is that of gravity.

The curved growth which takes place when slit stems are immersed in auxin solutions is of a somewhat different nature. In such curvatures, both the outer and inner parts of the slit organ are increased in growth rate, but the outer layers elongate most (82, 130). It was first suggested (130) that this is due to a failure of auxin to enter on the inner side, which is the wounded (slit) side, this failure being explained on the assumption that auxin can enter tissues only through intact epidermis. This assumption has since been disproved (170), and it is evident that although auxin reaches all the tissues freely, the curvatures are due to differences in the response of the two sides. This is supported by

additional evidence (170) that isolated inner and outer tissues of a stem respond to different ranges of auxin concentrations. Correspondingly, in a growing plant, with its own auxin supply, the tendencies of the different layers to grow are different, which causes a condition of mechanical strain or "tissue tension" well known to Sachs, Hofmeister and other early physiologists.

Cell division. High concentrations of auxins (about 1:10000) inhibit the elongation of stems and give rise to swellings. These swellings are in part due to enlargement of the parenchyma cells (83), but in part also they involve rapid cell division (106, 83, 101). In *Vicia* the extent of the swelling is approximately proportional to the logarithm of the auxin concentration (105). Removal of the roots greatly reduces the swelling (187). It occurs mainly in unphysiologically high auxin concentrations, but may become of importance in pathological conditions. Thus in galls (112) and intumescences (109) the parasite probably produces auxin within the tissues, the high concentration reached locally being largely responsible for the swelling.

Most of the histologically different types of cells are not stimulated to divide by auxin in low concentration (81), and the inference may be made that some other factor is limiting the process. The cambium, on the other hand, is activated to divide by pure auxins in concentrations comparable with those normally present, as Snow has shown (149, also 68), and the activity of cambium under normal conditions is thus due to auxin produced in the buds (199, 11). Other factors, particularly vitamin B_1 and bios (49), may also take part. Söding (153, 154) has shown not only that auxin production coincides with cambium activation in the spring, but also that in woody twigs the cambium is probably the main path of auxin transport. He even considers that auxin may be produced by the cambium. However this may be, the above facts evidently explain Jost's observation, made in 1891, that development of buds is correlated with sudden stimulation of cell division in the cambium below them.

The action of auxin on roots. The problem of hormonal control of root elongation was first approached through the effects of decapitation of roots on their subsequent growth. Even if the root tip does produce auxin (see section III), removal of the tip does not affect the growth of the roots of peas and some other plants, at least for 12 hours or so. The significance of auxin for the normal growth of roots is therefore questionable. Nevertheless roots are extremely sensitive to externally applied auxin. Indole-acetic acid in concentrations of 10^{-9} molal

(0.2 γ per liter) accelerates root elongation about 30 per cent (4, 60, 64, 58, 162), though apparently not in all plants, while higher concentrations inhibit elongation. High concentrations also cause thickening of roots (44, 164), but this is independent of the inhibition of elongation. The response of roots to auxin is thus an optimum curve, comparable with numerous phenomena of pharmacology.

The application of auxin to roots also causes branching, i.e., formation of lateral roots (162, 200, 83). This effect is comparable with the formation of roots on shoots. There is good evidence (164) that the well-known nodules on the roots of leguminous plants are due to auxin produced by the invading bacteria, the auxin first causing formation of a lateral root, and then inhibiting its elongation.

Enlargement of fruits. It was shown by Fitting in 1909–10 (61) that the swelling of the ovary, and other phenomena which occur after certain orchids have been pollinated, are due not to the fertilisation itself, but to some substance in the pollinia, or bundles of pollen grains. Alcohol or water extracts of the pollinia brought about similar effects. This action is probably due to auxin (102, 107), since (a) it is given by ether extracts of the pollinia or of urine, (b) the stimulating substance has the chemical properties of an auxin, and (c) direct *Avena* tests on the pollinia show auxin to be present in large amount.

The growth and swelling of fruits in presence of the pollen, but without fertilisation of the ovules, has been known since pre-Mendelian times, particularly in tobacco, but evidence that in such cases, other than orchids, it is due to a diffusible substance was not brought until 1934 (198). It was at the same time shown by extraction (160, 165) that auxin is present in varying amounts in the pollen of a wide variety of flowers. This suggests that auxin is the active agent, and the work of Gustafson (69) has shown this to be the case. Typical swelling was produced in numerous fruits, first by chloroform extracts of pollen and later by pure auxins. Small amounts of growth were also obtained in a few cases with substances which are not auxins, including oestrin and skatole, so that the effect is not highly specific, although good growth (comparable with that caused by pollination) was obtained only with true auxins. Doubtless the method will find horticultural application (cf. 63) in production of seedless fruits.

Bud inhibition. It was mentioned above that the development of buds is inhibited by auxin. In many growing shoots, the presence of the terminal bud inhibits the development of the lateral buds, and when the terminal bud is removed one or more of the laterals begin to grow.

It was shown by Thimann and Skoog (167) that the inhibiting power of buds goes parallel to their production of auxin, and if an amount of auxin comparable to that produced by the bud were applied to decapitated plants the laterals were completely inhibited. This has been confirmed for a variety of plants (103, 174, 117, 54, 65, 51) and also for a number of auxins. The growth of the buds, in so far as it consists of increase in size, is of course due to cell enlargement, and is presumably controlled by auxin. Correspondingly the beginning of development of the lateral buds (after the terminal is removed) is correlated with a large increase in the amount of auxin formed in them (167, 59, 128). This parallels the development of buds on trees in the spring. Thus auxin, which is essential for the subsequent development of the buds, at first inhibits their growth. How the same substance may both promote and inhibit bud development is not clear. The problem is apparently comparable to that of root elongation, which is promoted by low auxin concentrations and inhibited by higher ones, but it is much more complex. Numerous theories have been put forward (reviewed in 151), but it would be beyond our present scope to discuss them in detail.

Root initiation. One of the most important applications of hormone physiology is in the initiation of roots on cuttings. The pioneer work of van der Lek (111) and Bouillenne and Went (31) showed that root initiation is controlled by a substance formed in buds and leaves, and transported to the base of the stem (see also 121). At first it was believed that this substance is a special root-forming hormone, but it was soon found (165) that the richest source is in auxin concentrates from urine, or fungal cultures, and that in the course of purification, activity in causing root initiation went closely parallel to activity on growth of *Avena* coleoptiles. Finally it became clear that auxin itself is the active agent, pure auxin *b* (165) or indole-acetic acid (166, 85) causing vigorous root initiation. The fact that the transport of auxin is polar from apex to base of course explains the basal polarity of rooting under normal conditions. The rôle of other substances working together with auxin in the formation of roots will be discussed in section 6.

The earlier experiments (184, 165, 166, 104, 85, 68) were carried out by applying auxin to the apex of the cutting, and sugar solution or water to the base; the auxin was then transported to the base and caused rooting there. Under these conditions optimal rooting on etiolated pea cuttings resulted with indole-acetic acid at 2 to 10 mgm. per liter. However, certain auxins which are not readily transported

from apex to base were found (163) to cause very good rooting when applied at the base, although only weakly active when applied apically, and later it developed (48, 79) that all auxins may cause rooting when applied at the base, if only concentrations much higher than the above are used. As a rule 100 mgm. per liter of indole-acetic acid, or other of the highly active auxins, is the optimal concentration for rooting when applied basally, but the number of roots so formed can be very large. On account of its convenience in practice, this is the method which has been mainly used by horticulturists. A wide variety of woody and herbaceous cuttings give increased numbers of roots, increased percentage of rooting, and decrease in time of rooting, after auxin treatment. Concentrations of 25 mgm. per liter indole-acetic, indole-butyric or α-naphthalene-acetic acids or their salts, or possibly their esters, applied for 24 hours basally, are about the minimum. For woody material the application of sugar is generally not needed, on account of starch storage in the stem, though sugar may improve the subsequent maintenance of the cuttings (173). Those cuttings which do not root, or whose rooting is extremely scant, such as apples and pears and many conifers and forest hardwoods, generally are but little improved by auxin treatment. In these cases factors other than auxin must be limiting (see below). There is also a very marked age effect in such material (173).

VI. Factors other than auxin important for growth. Numerous investigations have indicated that there must be other substances besides auxins, which act in conjunction with the auxins in the regulation of plant growth. These other factors will now be considered, especially those which have been identified with known substances.

Carbohydrates. In his analysis of the growth of the *Avena* coleoptile, Went (182) showed that there must be some factor, necessary for growth, which comes directly from the reserves in the seed, in contrast with auxin which is applied to the growing region from the coleoptile tip. Towards the tip of the coleoptile, where there is auxin, growth should be limited by the amount of this second factor; in the base, where there is abundant supply of the seed factor, growth should be limited by the available auxin. This seed factor appears to be mainly carbohydrate. Schneider (140) has shown that the growth of coleoptile sections immersed in auxin solutions of optimal concentration can be increased by adding aqueous extracts of *Avena* seeds. He found, corroborating older observations (16) that pure sugar greatly increases the growth of such sections in optimal auxin, and further that the effect of seed ex-

tract can be completely replaced by 1 per cent sucrose solution. Levulose, dextrose and maltose are also effective, while mannitol has no such action (140, 169). Sugar, i.e., food material, is therefore a factor in the growth of the coleoptile. Sugar and auxin must be present simultaneously if growth is to occur.

The amount of available carbohydrate may also influence other actions of auxin than that on growth. Thus sugar is apparently essential for root development on cuttings treated with auxin (184). The application of auxin increases the rate of protoplasmic streaming in the coleoptile, and this effect also is dependent on the presence of sugar (159; see p. 547).

Osmotically active substances other than sugar. In addition to their functions as "food", the sugars serve to maintain the osmotic pressure of the plant cell. If no fresh supply of osmotically active material were available, growth by the uptake of water would necessarily soon cease. Thimann and Schneider (169) have found that the growth of coleoptile sections in optimal auxin concentrations is promoted not only by sugar but also by neutral salts, especially KCl. The combination of sugar and salts is better still. Thus while sugars influence growth of the *Avena* coleoptile primarily through their function as "food factors", they may also act in part merely through their effect in maintaining osmotic pressure, and it is in this latter function that sugar may be replaced by inorganic salts which permeate the cell readily. In the chlorides the order of effectiveness is $Ca < Li \lessgtr Rb < Na \lessgtr K$.

Factors for root growth. It has been shown above that while very low concentrations of auxin may cause small increases in root elongation, still the typical effect of auxin on roots is inhibition. Whether or not auxin is essential at all for root growth cannot as yet be definitely stated. That special growth hormones of quite a different nature are required for root growth has, however, been established through investigations of the growth of roots in culture. Tips from the roots of seedlings may be grown indefinitely *in vitro* (under sterile conditions) in a suitable nutrient medium. Thus White (190, 191) cultivated tomato roots through weekly passages for several years. His medium contained 0.01 per cent yeast extract in addition to sucrose, nitrate and the usual inorganic constituents. The yeast extract is apparently essential, not only for tomato but also for pea roots (22, 24). The substance in yeast extract which is of primary importance for growth of pea roots has been shown by Bonner (22) to be vitamin B_1. Vitamin B_2 (lactoflavin) was without significant effect (24), as was (23) pantothenic acid (195).

Robbins and Bartley (135) independently have found vitamin B_1 essential for the growth of excised tomato roots, and their work has been confirmed (193). Vitamin B_1 is in fact a general growth factor for roots of higher plants (188). There is abundant evidence in the older vitamin literature that vitamin B_1 is formed in green leaves in the light and is stored in seeds (80, 21). Thus vitamin B_1 is to be considered as a plant growth hormone, since it is formed in one part of the plant and transported to another part. It affects root growth primarily because it is essential for cell division in the root meristem (1).

Vitamin B_1 alone, however, will not support long-continued optimal growth of pea or tomato roots *in vitro*. Certain amino-acids must also be supplied to the tomato root, viz., glutamic acid, phenylalanine, lysine, histidine, proline, valine, serine, leucine and isoleucine (192). In the pea root, although vitamin B_1 is able to support continued growth at a diminished rate (0.5 cm. per day) the growth rate can be increased up to 1.2 cm. per day by the addition of small amounts of certain amino-acids to the medium (24). Apparently the root can synthesize the bulk of its amino-acid requirements from nitrate, but some "specific" amino-acids are synthesized in such small amounts that growth is benefited when they are supplied. Such a situation would resemble that reported by Rose (137) for the arginine requirement of the rat.

Indirect effects of vitamin B_1. Since auxin, vitamin B_1 and presumably other plant growth substances or their precursors are formed in green leaves in light and are stored in the seed, the most general test for plant growth factors should be one in which the entire plant is deprived as far as possible of these sources of growth substances and kept in the dark. A favorable object for such investigations has proved to be the embryo, excised from the seed and grown on nutrient medium *in vitro*. Pea embryos have been used extensively; they are removed from the cotyledons (which contain reserves both of food material and of special growth factors) and cultivated under sterile conditions in the dark. If a medium containing only sugar and mineral salts is used, the embryo grows into a dwarf plant about 15 mm. tall after 3 weeks (25, 93). That even this small amount of growth takes place is undoubtedly due to small amounts of growth factors present in the embryo at the time of excision (23). However, if vitamin B_1 is added to the medium in small amounts (0.001 mgm. per embryo), not only is the growth of the root increased, (93, 25) but the final length of the shoot may also be increased by 100 per cent or more. Vitamin B_1 has no beneficial influence on the growth of isolated shoots *in vitro* (23) and it

seems likely that it increases shoot growth indirectly through its effect upon the root. A possible mechanism for this effect is suggested by the fact that when vitamin B_1 is applied to the roots of pea embryos it greatly increases the synthesis of vitamin C in the shoot (26). Vitamin C is also a growth factor for the shoot (see below). Lactoflavin and nicotinic acid are additional substances of the vitamin B complex which are beneficial to the growth of these isolated embryos (23). They apparently act specifically on bud growth rather than on root development.

Biotin; pantothenic acid. Kögl and Haagen-Smit (93) showed that crystalline "biotin" (the bios II factor for yeast growth) (97) increases the growth of the shoot in the pea embryo test. The amount of biotin which must be supplied to an embryo for maximal effect is roughly equal to that which is normally present in the pea seed. Biotin is thus apparently stored in the seed as a growth factor for the seedling, and is also to be regarded as a plant hormone. Pantothenic acid (195), another bios II factor closely related to biotin, exerts an influence on the growth of the pea embryo (25) similar to that of biotin.

Pure biotin, in conjunction with auxin and sugar, also increases the number of roots upon pea cuttings (186). The effect is probably upon the initiation of the roots, rather than on their subsequent growth, since pantothenic acid, which would be expected to have the same effects as biotin, has no effect on the growth of pea roots (23).

By testing for bios II on yeast growth, Dagys (49) has found that this factor is "mobilized" from the reserve of the seed by the growing seedling during germination. In buds as they develop in spring there is a large increase of bios II (49), and in general, high growth activity of buds and leaves seems to be correlated with high content of bios II.

Vitamin A. Other vitamins besides B_1 possess physiological activity in the plant. Thus carotene (pro-vitamin A) is reported (110) to increase the number of roots formed on cuttings of *Impatiens.* A relationship between carotene and phototropic response (12, 42) is indicated by the fact that the absorption spectra of the carotenoid pigments from the *Avena* coleoptile (37) and from the sporangiophores of Pilobolus (36) coincide closely with the spectral distribution of phototropic sensitivity of these organs. The connection may be brought about (99) through the action of carotenoids as "sensitizers" of the photoinactivation of auxin on the illuminated side of the organ.

Ascorbic acid. The growth of seedlings of some plants is benefited by the application of ascorbic acid (73, 74, 50). However, different

species vary greatly in their response to this substance (74) and even within one species (the pea) conflicting results have been reported (93, 25). It is now clear that these discrepancies are caused by differences between even closely-related plants in the ability to synthesize ascorbic acid. Thus, of 9 different pea varieties whose embryos were cultivated *in vitro* as described above (26), four responded to the addition of ascorbic acid with increase in shoot growth, four were unaffected, and one was intermediate. The four varieties which did not respond were shown to synthesize 3 to 5 times as much ascorbic acid as did the four which did respond. Thus the varieties which did not respond to added ascorbic acid were apparently able to cover their ascorbic acid requirements by their own synthesis.

Growth factors influencing green plants. Even green plants growing normally in light, and thus free to synthesize all needed growth substances, may be limited by a particular internal factor. Indeed, it might be expected that plants growing under optimal external conditions should be limited by some internal condition. Thus, the growth of a liverwort may be increased by the application of pantothenic acid in small amounts (196) and the same has been shown for alfalfa (115). Many green plants, particularly varieties noted by gardeners as being "slow growing" appear to be limited by the amount of vitamin B_1 available (23). The shoot growth of such plants has been increased by the addition of vitamin B_1 to the nutrient solution in sand cultures, or by merely watering plants growing in soil with dilute vitamin B_1 solutions.

Oestrogenic substances. In recent years many investigators have studied the effects of oestrogenic substances on plant growth. Oestrogenic substances actually occur in plant material (113, 143, 39). They do not, however, regulate the reproductive activity of plants as was earlier supposed (113, 142). Scharrer and Schropp found (139) that corn, wheat, soy beans, red clover and alfalfa responded with increased vegetative growth to the addition of follicle hormone under different conditions. Oestrogenic substances are stored in the pea seed, and crystalline follicle hormone has a beneficial effect on the growth of the pea embryo *in vitro* (93, 25). Hence some at least of these substances may have to be considered as plant hormones.

Wound hormones. It was shown many years ago by Haberlandt (71) that when plant cells are wounded there are liberated substances capable of causing renewed growth activity in other, uninjured, cells. Such "wound hormones" would of course function in the healing of

wounds. Many different tissues exhibit a response to preparations of wound substances, e.g., discs of potato and of kohlrabi root (71), leaves of succulents (71), petioles and stems (134, 194) and the seed chambers of the Phaseolus (string bean) pod (179). They have also been used for the maintenance of the growth of undifferentiated tissues *in vitro* (20). The chemical nature of the active substance has been extensively investigated only in the case of the string bean pod, in which the wound hormone reaction consists of a small neoplasm-like knob of tissue, which grows out of the inner lining of the pod when a drop of solution containing the active wound hormone is applied. Umrath and Soltys (175), using a semi-quantitative test with bean pods as criterion of activity, fractionated alfalfa hay, and obtained an impure acidic product active at a dilution of 1:50000. Bonner and English (27) have described a quantitative method for assaying the bean wound hormone, in which the activity is measured by the volume of new growth, which, with certain reservations, is proportional to the amount of wound hormone applied. Using this test, they obtained, by fractionation of bean pods (57), an amorphous acidic product with molecular weight approximately 220 and active at a dilution of 1:100,000. A comparison of these two products would be of little value since neither was pure. However, the bean wound hormone is apparently not identical with any other known plant growth factor (27). Tyrosin, found active as a wound hormone (122) in Haberlandt's kohlrabi test, is without effect upon bean pods.

The interaction of growth factors. In the earlier work on plant hormones, there was perhaps a tendency to emphasize the importance of auxin to the exclusion of all other factors. It is now clear, however, that plant growth, like all other biological processes, is controlled by many substances. In one particular plant or organ, and under one particular set of external conditions, one of these substances may be more nearly a "limiting factor" than the others. Under the conditions of the *Avena* test, for example, growth of the coleoptile is primarily limited by auxin. However, the amount of available carbohydrate (140) or of salts (169) may also limit the growth of the *Avena* coleoptile, and in fact auxin and sugar may simultaneously be "limiting factors" (140) so that an increase in either results in an increase of growth. Similarly, in the excised pea embryo, growth is limited primarily by available "foods", e.g., sugars, nitrate, etc., but, in presence of optimal amounts of these, the further growth of the embryo is limited by the specific growth factors,—biotin or pantothenic acid, vitamin B_1, vita-

min C, oestrone, vitamin B_2 and nicotinic acid (93, 25, 23) and perhaps others. Here again the action is not one of simple "limiting factors" in the sense of Blackman (29), but rather of interacting factors, the increase of *any one* of which results in an increase of growth.

The necessity for the coöperation of several hormones for the production of a single growth effect is well illustrated in the rooting of cuttings. If a cutting is to root, it is necessary first that root primordia be initiated, and then that these primordia grow out into functional roots. For the first process, auxin is essential (see section V). It may possibly interact with other as yet unidentified internal factors (48, 187). The further growth of the primordia, however, requires quite other substances. Available sugar is necessary (184, 186) just as for the growth of roots in culture (190, 24). In addition, vitamin B_1 is probably essential for root growth on cuttings, although it is without effect on the initiation of root primordia (188). The rooting of cuttings of *Camellia*, *Dracena* and some others is strictly limited by the available vitamin B_1 (188, 23). It is probable that vitamin B_1 is the "factor necessary for the growth of roots" which is produced (48) in the leaves of leafy cuttings.

VII. The mechanism of auxin action. *The importance of transport.* As stated on p. 526, an essential part of the concept of a hormone is that it is produced in one region and exerts its effect in another. Only in this way can one part of the plant control the development of another. The hormone may also, of course, act in the region in which it is produced, but this in no way affects the importance of the control of other parts. With the auxins such control is always exerted in a basipetal direction. An exception is provided by certain types of shoot inhibition, in which two growing shoots inhibit one another even though the inhibiting influence must travel down one shoot and up the other in order to exert its effect. In this case, however, it is by no means certain that the inhibition is due only to auxin.

In isolated stems and coleoptiles, auxin applied at the apical end diffuses out at the base, even if the agar into which it diffuses already contains a higher auxin concentration than that applied (181). Such transport "against the gradient" indicates an active carrying process quite other than diffusion. Application at the base, on the other hand, causes little or no diffusion out of the apical end. If there is acropetal transport it is only a small fraction of that in the basipetal direction (150), or else it begins only after a high threshold concentration has been reached (185). The polarity of transport is dependent on some

vital process since it may be temporarily suspended by narcotization with ether vapor (181).

Transport of externally applied auxin may, however, take place acropetally if there is transpiration (150, 78). Skoog has shown (146) that auxin is drawn upward in the transpiration stream in the xylem, just as salts, dyes and other compounds may be, but as it moves upwards it passes out into the living tissue and is in this again transported downwards. This downward transport is of the usual polar type. If the stem is ringed, upward movement in the xylem still occurs, but on passing downward the auxin accumulates above the ring (146, see also 48, 35). This is perhaps the nearest approach to a circulation yet established in plants.

The effect of pH is of interest in this connection. Mineral acid alone may cause curvature or acceleration of straight growth in *Avena* coleoptiles under certain conditions (158, 17). This effect of acid runs parallel to its effect on dissociation of the auxins, when allowance is made for the discrepancy between external and internal pH (17). The pK of indole-acetic acid is about 4.7 (53, 2), and that of auxin *a* is not very different (100), while the pH of coleoptile sap is close to 6 (17). Hence most of the auxin is present in salt form. The "acid growth" is therefore due to the setting free of auxin acid from its salts, since the undissociated acid penetrates much more readily through cell membranes than the salts (3, 169), and therefore passes more rapidly from cell to cell.

Primary and secondary properties. The *Avena* curvature method depends, of course, on polar transport, and also on the relative unimportance of lateral transport, since if the auxin were to move across to the opposite side more rapidly than it moved downward the growth of both sides would be promoted and there would be no curvature. Nevertheless, this is probably just what happens in the case of benzofurane-2-acetic acid (163), which produces no curvature but can promote straight growth. Several substances, including indene-3-acetic acid and indole-3-butyric acid, give only weak curvatures when applied in agar to *Avena*, but can still produce maximum stimulation of straight growth when applied in solution. Here too there is evidence (163, 8) that their transport downward is less rapid than their movement laterally.

It is evident that the activity on coleoptile or stem sections immersed in auxin approaches the true growth-promoting activity of the substance, uncomplicated by transport factors. Such uncomplicated

growth promotion has been called "primary" activity (186, 84). If the influences of secondary factors are excluded as far as possible, the activities of many auxins per molecule, corrected to the undissociated auxin acid, are approximately the same (14). This seems to indicate (cf. 186) that auxins react in a stoichiometric fashion, i.e., react mol for mol with an as yet unidentified cellular constituent. Under the usual test conditions some secondary factors are always operating; these include the rate of inactivation of the auxin, the dissociation constant of its acid group, the rate of entry into the cells, and the concentrations of factors other than auxin present. It must be for this reason that even if the physiological conditions are identical, i.e., with straight growth of immersed sections, the relative activities of two given substances still differ with the plant used. Thus, using concentrations in the range of proportionality, indole-3-butyric acid has 22 per cent of the activity of indole-acetic on the growth of pea stems, but only 9 per cent of the activity of indole-acetic acid on the growth of oat coleoptiles. The corresponding figures for indole-propionic acid, also compared to indole-acetic as standard, are 8 per cent and 0.8 per cent respectively (173). Thus the spread between the activities of different substances is greater in the coleoptile than in the pea stem.

The relative activities of different auxins not only differ in different plants, but also, apparently, in two different tissues of the same plant (170).

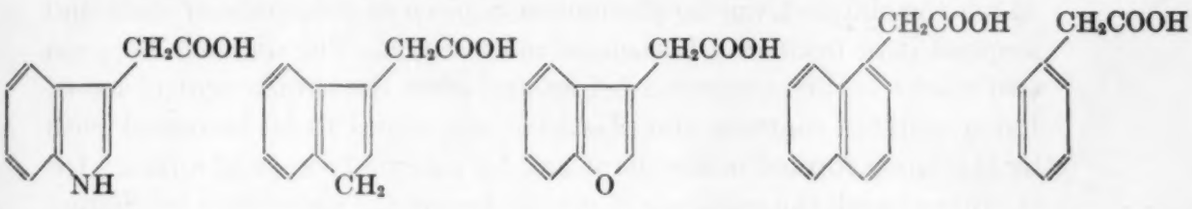
An explanation of these differences in relative activity can readily be suggested. For instance, indole derivatives are unstable in acid solution, while auxin *a* is unstable in alkaline (91). On a plant or tissue with very acid internal pH, the activity of auxin *a* would thus appear higher than that of indole-acetic acid and *vice versa*. Similarly auxin *b* is more sensitive to H_2O_2 than indole-acetic acid (165) and indole-acetic acid is more sensitive than indene-acetic acid (163), while auxin *a* is more sensitive to the plant oxidases than indole-acetic acid (125). The oxidising powers of a tissue or organ may therefore influence relative activities (cf. section IV). In the inhibition of root elongation this is of particular importance, because roots are very active in oxidising auxin (66, 60, 129).

The relation between structure and activity. A group of substances of such relatively simple nature as the auxins is very suitable for an attempt to determine what structural elements are necessary for activity. As mentioned above, the auxins are all unsaturated acids, and Kögl, Haagen-Smit and Erxleben (87) showed that the dihydro-derivatives

of auxin *a* and *b* and indole-acetic acid (94) are inactive on *Avena*, so that the double bond is apparently essential. No saturated substance yet tested has shown auxin activity. Introduction of methyl groups reduces the activity greatly (95). As regards length of side-chain, there is a very suggestive alternating effect (186, 95). If I represents the indole nucleus, the activities, in relative values, are:

ICOOH, 0; ICH_2COOH, 100; $I(CH_2)_2COOH$, 0.1;
$I(CH_2)_3COOH$, 5–10; $I(CH_2)_4COOH$, 0.1

The *Avena* test, however, being greatly influenced by transport and perhaps other secondary factors, is much more selective than the curvature of slit stems. This is well shown by the acetic acid derivatives of different nuclei (84, 163, 170):



Activity on immersed slit pea stem	100	20	15	>100	2–10
Activity in agar on *Avena* coleoptile	100	1	<0.1	2	0.02

The decrease in activity on passing from naphthalene-acetic to phenyl-acetic is paralleled by a similar difference between the activities of indole-acetic (very high) and pyrrole-acetic (very low) (173). The additional benzene ring thus makes an important contribution.

Using the curvature of slit pea stems, Koepfli, Thimann and Went (84) have deduced the following requirements for primary activity: *a*, a ring system; *b*, a double bond in this ring; *c*, a side-chain; *d*, a carboxyl, —or a structure readily converted to a carboxyl,—on this side-chain at least one carbon atom removed from the ring, and *e*, a particular space relationship between the ring and the carboxyl group. The evidence for *b* is that 2 compounds with a saturated ring and a double bond in the side-chain, but all other requirements fulfilled, are both inactive. The evidence for *e* is the fact that *cis*-cinnamic acid (70) and its *p*-methyl derivative are active, while the *trans*-forms are not. The molecule must presumably be bent so that the distance between the carboxyl and the ring (or possibly the double bond in it) falls within a certain

range of values. This may explain the alternating activity with increasing length of side-chain. In general it is of interest that activity is not conferred by any one group, but by a certain interrelationship of groupings in space.

The physiological approach. Two methods are available for the elucidation of the mechanism of auxin action. One is the study of the structure of the active molecules, discussed above. The other is the analysis of the reactions involved in the growth process. Part of this latter has centered about the cell-wall, part about the cell contents.

Heyn (77) and Söding (152) have shown that the plasticity of the wall, as measured by the irreversible stretching under a given load, is increased in the presence of auxin. This was done in several ways, of which the simplest was to plasmolyze a piece of coleoptile or stem and suspend it in front of a horizontal microscope. The distance between two marks is then measured before and after the attachment of loads. Using suitable controls, the plasticity was found to be increased both by the auxin formed in the plant and by externally applied auxin. On the other hand, the existence of certain important exceptions led Söding to conclude that the plasticity change cannot be the primary cause of growth. A claim that dead plant material of several kinds shows increase in plasticity on treating with indole-acetic acid (136) has been disproved (157).

Analyses of the cell-wall material of young coleoptiles (168, 120) show that it contains pectins, hemicelluloses, a considerable amount of protein, and cellulose. There is also a wax (120, 197), the amount of which in young material may be so great as to obscure the x-ray diffraction due to cellulose (76). On account of its crystalline nature, however, the cellulose is the constituent which has been most studied. There is a good deal of evidence that it exists in cell-walls as micelles, or rod-shaped bundles of macro-molecules (see 62). An increase in plasticity would therefore be due to a release of the forces binding the micelles together. This would then allow the micelles to be forced apart by the osmotic pressure of the cell contents: "growth by stretching" (62). Optical methods, however, indicate that the orientations of the cellulose micelles (18) and of the wax particles (197),—which are both mainly in a direction perpendicular to the axis of elongation,—do not change during a large part of the growth of the coleoptile. Hence, if the micelles become temporarily stretched out in the direction of elongation, they must be continually sliding back into their original lateral orientation (62), or else they must be continually over-

laid with new micelles having the lateral orientation. The evidence is as yet insufficient for a decision on this point.

As to how the forces between micelles could be decreased by auxin is not at all clear. Any kind of adsorption to the micelles is negatived by the calculation that there are not enough molecules of auxin present to be adsorbed one to each micelle (168), even if every auxin molecule introduced were effective. Rüge (138) considers that the volume of the amorphous material lying between the micelles might be increased by some sort of swelling process, but the evidence is not convincing. All in all, though many facts have been accumulated, and the study of cell-walls greatly stimulated, conclusions as to the method of growth are still uncertain.

As regards the cell contents, it must first be remembered that the action of auxins,—especially when accompanied by sugar, salts and the other co-factors,—is a very general one, and not limited to cell elongation. According to the tissue and to the conditions, growth promotion, growth inhibition, cell division or root initiation may be caused. Auxin even promotes bleeding from a cut surface (189, 148). Presumably "all these functions arise from one primary reaction in the cell; which physiological effect is produced depends upon the nature and position of the tissues affected. The actual primary reaction .... is a kind of master reaction governing the activities of the cell" (163). In keeping with this idea, there is much evidence associating the action of auxin with an oxidation process. Elongation does not take place in nitrogen (15). Further, phenyl-urethane or KCN reduce the elongation of coleoptiles in auxin, and concentrations of KCN which cause partial reduction in growth rate cause about the same percentage reduction in respiration rate (19). Observations on protoplasmic streaming establish an even closer connection between auxin and oxidations. The streaming in the epidermis of the *Avena* coleoptile is extremely sensitive to oxygen tension (30). When auxin is added the streaming rate is increased by those concentrations which accelerate growth, and inhibited by concentrations which inhibit growth (172). Several auxins act in the same way. After about 30 minutes the rate returns to normal, but if sugar be added the increased rate is maintained (159). Hence auxin probably accelerates an oxidation process of which sugar is the substrate, and this process is doubtless linked with the growth acceleration. The fact that pure auxin has not been found to increase the respiration of coleoptile sections detectably (92, 19) indicates that the oxygen consumption of the auxin-sensitive oxidation process is only a

small fraction of the whole respiration. This is supported by the observation (159) that when the total respiration is increased by dinitrophenol this has no effect on growth. Thus auxin probably catalyzes one specific respiratory process. This agrees not only with the evident importance of sugar, both for growth and for protoplasmic streaming, but also perhaps with the rôle of vitamin B_1 as a cofactor, for vitamin B_1 is known to play a part in oxidations via co-carboxylase. Further, if the specific process catalyzed also controls protoplasmic streaming, this, by promoting interchange of materials within the cell, could lead to a variety of final effects both upon the wall and upon enzymatic processes, and so act as a "master reaction" of the type specified above. It must be admitted, however, that these views are still partly hypothetical.

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THE PHYSIOLOGICAL EFFECTS OF SMALL AMOUNTS OF LEAD: AN EVALUATION OF THE LEAD HAZARD OF THE AVERAGE INDIVIDUAL

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The amount of lead absorbed and retained in the body even in fatal poisoning is usually less than a gram and hence might be spoken of as small. It is not our purpose, however, to add to the great numbers of papers and numerous reviews (Tanquerel des Planches, 1839; Meillère, 1903; Legge and Goadby, 1912; Oliver, 1914; Aub, Fairhall, Minot and Reznikoff, 1926) of the familiar picture of chronic plumbism. Here we are concerned rather with the more insidious effects of those quantities of lead to which the average individual living in modern industrial society is exposed. We must therefore:

1. Estimate as accurately as possible the magnitude of the lead exposure of the average individual;
2. Establish the quantitative relationship of this amount of lead to that which is recognized as likely to cause outspoken plumbism; and
3. Review what is known of the mechanism of the physiological action of small amounts of lead in the organism in order to evaluate the possible hazard of an exposure to lead insufficient to cause the commonly recognized signs and symptoms of poisoning.

While no entirely satisfactory conclusion can yet be arrived at with regard to any of these points, the purpose of the following discussion is to bring together the pertinent information which is available at present.

THE LEAD EXPOSURE OF THE AVERAGE INDIVIDUAL IN MODERN LIFE. *General approach to the problem.* The early recognition that lead causes poisoning has gradually brought about regulations which minimize the possibility that the average person in modern society will be accidentally exposed to gross amounts of lead. At the same time, however, certain modern practices make probable a constant exposure of

the general population to minute amounts of lead. Tremendous amounts of lead are used annually in insecticides and are added in the form of tetraethyl lead to gasoline used for motor fuel. As these products are used the lead is scattered in finely divided form over vegetation, top soil and surface waters. Such a dissipation of lead, as well as the modern custom of preserving a large fraction of our food in metal containers, must make it possible for the average individual to absorb small amounts of lead in dust-laden air and in the food and water consumed. The problem of estimating the significance of this small constant exposure may be attacked either from a study of the factors contributing to the total lead exposure or from a study of individuals who have lived for some time exposed to such sources in an average normal environment. The first approach would involve determination of the lead content of typical diets, water supplies, samples of air, etc., while in the latter the attention would be turned to a study of the lead in the excreta, blood and tissues of persons without industrial exposure to lead.

Chemical methods. Whatever the approach, the first requisite is a chemical method which is delicate and at the same time specific. Minute amounts of lead must be separated from relatively huge amounts of other material and then accurately measured. There are several methods advocated for the detection of small amounts of lead. These include two variations of the measurement of lead separated as chromate from a solution of ashed material. One is the titration method described by Fairhall (1922) and the other a colorimetric method based on a reaction between chromic acid and s-diphenyl carbazide described by Cazeneuve (1900). Great sensitivity has been claimed for the colorimetric method but Fairhall (1922, 1933) has thrown some doubt on its reliability. However, it has been extensively used in a somewhat modified form by Kehoe and his associates (Kehoe, Edgar, Thamann and Sanders, 1926) and others. The so-called dithizone test is another method for which great accuracy and specificity are claimed. The test is based on a reaction described by H. Fischer (1929) between lead and diphenylthiocarbazone. A colorimetric adaptation of this method is described by Bohnenkamp and Linneweh (1933) and a titrimetric procedure is advocated by Howitt and Cowgill (1937). The spectrographic method (Shipley, Scott and McNair, 1932) is also frequently employed for the detection and estimation of extremely small amounts of lead. Besides these quantitative methods, Fairhall (1923) has described a qualitative microchemical test based on the formation of crystals of complex potassium-lead-copper-hexanitrite which can be

recognized by their characteristic form. This test is extremely useful as a supplementary proof of the identity of small amounts of material which are indirectly measured as lead by other methods.

Lead in tissues and excreta of normal individuals. Our present information as to the magnitude of the exposure of the average individual to lead must be based on data gleaned from numerous sources in which the problem has been approached from various points of view and by the use of different chemical methods. Aub and his associates (1926), using Fairhall's method, found no lead in the urine and tissues of persons with no unusual exposure to lead. Even in the skeleton, where lead is most likely to be stored, none was usually detected except in individuals in whose history some unusual exposure to lead was revealed. These authors concluded that at that time in their locality (Boston) "the usual activities of daily life cause no retention of lead in the body." At about this same time Kehoe and his co-workers (1926) in Cincinnati, using the diphenyl-carbazide test, found lead present in the excreta of 65 normal persons and concluded that the general finding of lead in the urine must indicate that nearly everyone has absorbed some lead. Since that time numerous investigators, including Barth (1931), Weyrauch and Müller (1933), Pfrieme (1934), Maulbetsch and Rutishauer (1936) and Tompsett (1936), working with a variety of chemical methods and in different localities, have found lead present in the bones of practically all normal individuals without history of unusual exposure to lead. The concentrations reported ranged from about 0.5 to 10.0 mgm. Pb per 100 grams of ash. Usually the higher concentrations were found in the bones of older individuals and no consistent difference was noted between the lead contents of bones of city dwellers and of those who had spent their lives in rural communities.

More extensive surveys of normal lead exposure. In a typical modern American industrial city, Kehoe, Thamann and Cholak (1933a) studied the lead content of foods and of the excreta, body fluids and tissues of persons without industrial exposure to lead. It was found that the mean daily output of lead by normal adults was about 0.25 to 0.30 mgm. Most of this was found in feces, but the urine contained from 0.02 to 0.08 mgm. per liter. Lead was also found in the excreta of infants and children (Kehoe, Thamann and Cholak, 1933b). Human milk was found to contain from 0.0 to 0.05 mgm. of lead per liter. From studies of a large variety of foods, the authors conclude that the average daily consumption of lead by an adult is about 0.20 to 0.35 mgm., as compared to a daily intake of 0.10 mgm. by individuals living under

primitive conditions (Kehoe, Thamann and Cholak, 1933c). In persons in both primitive and modern communities, the daily excretion of lead was practically the same as the estimated daily intake. Some of the ingested lead must have been absorbed, since it was detected in the urine, blood and tissues. Even stillborn fetuses were found to contain small amounts of lead. The tissues of a child of two years were shown to contain approximately the same concentrations of lead as those of an adult, although the total present in the body was greater in the older individual.

It is of interest to compare these results with those of Tompsett and Anderson (1935), working at the Royal Infirmary in Glasgow. The latter authors used the dithizone method which was apparently carefully controlled. Analyses were made of the tissues obtained at autopsy from 20 persons without occupational exposure to lead. The ages ranged from 2 to 69 years. Lead was found in all the tissues analyzed. Stated in terms of milligrams of lead per kilo of fresh tissue, the following average concentrations were found: liver, 1.73; kidney, 1.34; spleen, 1.69; brain, 0.50; rib, 8.55; vertebrae, 7.09. The tissues of a child of two years were found to contain approximately as high concentrations of lead as did those of adults. Lead was also found in smaller but appreciable amounts in the tissues of 4 fetuses of 7 to 8 months' gestation. Analyses of blood from 3 normal persons and 18 patients (without exposure to lead) gave values ranging from 40–70γ with an average of 55γ of lead per 100 cc. The mean daily lead excretion of 10 patients was found to be 0.05 mgm. in the urine and 0.22 mgm. in the feces. This daily output was in close agreement with the estimated intake based on analyses of food and water. The average daily diet contained about 0.22 mgm., while the water had a lead content of 0.03 mgm. per liter.

Sources of "normal" lead. A few studies are available which furnish some idea of the relative importance of various sources which contribute to the total incidental exposure to lead in a given locality. Arranged in order of increasing importance as dietary sources of lead (Kehoe, Thamann and Cholak, 1933d) are listed: breadstuffs, meats, processed meats, ice cream, candy, leafy green vegetables and certain fruits. The relatively high lead content of vegetables and fruits is probably at least partly due to the general use of insecticides. Lendrich and Mayer (1927) found from 0.0 to 4.0 mgm. of lead per 3 apples (the sample uniformly chosen for analysis) raised in the United States. Much of this lead was on the outside of the fruit. No lead was found in apples

grown in Australia, where lead is not extensively used as an insecticide. Little data are available as to the importance of inhaled lead as a part of the total exposure. Bloomfield and Isbell (1933) report an average of 0.09 mgm. of lead per 10 cubic meters in 28 samples of air collected at congested street intersections and slightly higher concentrations in the air of industrial plants not handling lead, and in automobile repair shops.

General conclusions. When reviewed as a whole, the uniformity of results, obtained by numerous investigators in widely separated parts of the world and by means of a variety of chemical methods, leaves no doubt that the average individual in modern civilized communities continually ingests or inhales small amounts of lead. The closely agreeing results of Kehoe and his associates in the United States and of Tompsett and Anderson in Glasgow indicate that the magnitude of the average daily incidental exposure is probably from 0.2 to 0.4 mgm. of lead. Excretion more or less keeps pace with the intake, so that no marked increase in the concentration of lead in the body occurs with age. Some lead is, however, retained and may be detected in the blood and soft tissues, although the greater proportion of retained lead is found stored in the skeleton and teeth.

QUANTITATIVE RELATIONSHIP OF AVERAGE GENERAL EXPOSURE TO LEAD TO THE EXPOSURES WHICH RESULT IN OUTSPOKEN PLUMBISM.

Estimates of amounts of lead causing poisoning. Sollmann (1922) estimates that the intake of lead which usually eventually causes clinical plumbism is about 10 to 20 mgm. per day for an adult (0.2 to 0.4 mgm. per kilo) but suggests that smaller amounts probably do some harm. Numerous other investigators place this estimate much lower—as low as 1.0 mgm. per day for adults. Wright, Sappington and Rantoul (1928) report occasional poisoning in persons taking as little as 0.1 mgm. of lead daily in drinking water over a period of years, while poisoning was common in individuals taking 1.5 mgm. per day. Studies which measure only one of the possible incidental sources of lead may result in erroneously low estimates of the amounts of lead which produce signs of plumbism. It is evident, however, that attempts to predict the danger of a given exposure to lead solely on the basis of the amounts involved results at best in only a rough estimate.

Factors which influence individual susceptibility. Whenever the toxicity of lead compounds is investigated a wide difference in individual susceptibility is noted. Some persons can work with impunity for years at the same lead trade in which others quickly develop signs of poisoning.

Even when experimental animals are carefully selected with regard to uniformity of age, sex, previous nutrition, etc., and are exposed to the same amounts of lead there is a wide variation in the time of appearance and the severity of symptoms of poisoning. On the other hand, when single tissues such as blood cells, frogs' muscles, intestinal strips, etc., are exposed to lead for the purpose of studying some particular action of the metal, the results are much more uniform. Under these simpler conditions there is usually a consistent quantitative relationship between the amount of lead present and the intensity of the effect produced. Individual differences in susceptibility are, therefore, probably due not so much to an essential difference in the resistance of tissues to the effects of lead as to variations in the factors which influence the amount of soluble lead to which the tissues are exposed. Such factors include the absorption, storage, mobilization, and excretion of the lead which gains entrance to the body; and each factor is important in determining the danger of a given exposure to lead to each individual.

Absorption of lead. Fat-soluble organic compounds such as tetraethyl lead are readily absorbed through the skin (Flinn, 1926), but for the type of lead to which the ordinary individual is exposed only the gastro-intestinal and respiratory tracts need be considered as portals of entry.

Even on heavy exposure, by no means all of the lead taken into the gastro-intestinal tract is absorbed. Both the solubility of the compound ingested and the possible formation of non-diffusible compounds with other materials present in the gastro-intestinal tract influence the completeness of absorption. The portion which is absorbed is carried in the portal blood to the liver, where much is removed from the blood and held back from the general circulation, as evidenced by the higher concentrations of lead in the liver of animals receiving lead by mouth than when similar amounts are introduced by other routes (Minot, 1924a). Some of the lead which reaches the liver is excreted in the bile and probably never reaches the general circulation; some, however, is distributed to the other tissues of the body.

Minot (1924b) has stressed the more rapid entrance of lead to the general circulation when it is introduced into the respiratory tract. Blumgart (1923) has shown that lead need not reach the lungs but that ready entrance to the blood stream is afforded to lead in the upper respiratory passages. Lead absorbed from the respiratory tract enters the systemic blood directly and so is promptly distributed throughout

the organism. Hence, although the amount inhaled may be much less than that ingested, it is potentially more dangerous.

Distribution of retained lead. Once in the general blood stream, the distribution of lead follows a similar pattern whatever the portal of entry has been. All the tissues receive some lead. Higher concentrations are usually found in bones, liver, kidney and spleen than in other organs. Excretion more or less keeps pace with retention in the soft tissues of the body, so that there is no great increase in the concentration of lead even during periods of continued absorption. The bones store lead and continued absorption results in a progressive increase in the concentration in the skeleton and teeth.

Biochemical behavior of lead in the body. There is not complete agreement as to the biochemical behavior of lead in the organism. The extensive studies of Fairhall and Shaw (1924), Aub and Reznikoff (1924), Brooks (1927), Bischoff, Maxwell, Evans and Nuzum (1928) furnish rather convincing evidence that in whatever form lead is absorbed it is converted to inorganic phosphates. According to the findings of Fairhall and Shaw (1924), as interpreted by Aub and his co-workers (1926), lead is probably transported in the blood stream as the more soluble di-lead phosphate and is stored in the bones as the very insoluble tertiary phosphate. Maxwell and Bischoff (1929a) and Kehoe and Thamann (1933) hold that other forms of lead more active than the phosphate are present in blood. Work by Maxwell and Bischoff (1929b) indicates that lead is carried in the blood as the diphosphoglycerate, while Jowett (1932) presents evidence that lead forms a complex inorganic phosphate containing calcium and chlorine. As Aub (1935) points out, it is possible that several compounds of lead exist in so complex a chemical medium as blood plasma.

Whatever the form in which lead is transported, it is the lead in the blood and body fluids which can do injury to body cells, while lead stored in the bones is relatively harmless. The deposition of insoluble tertiary lead phosphate in the bone is, however, not an irreversible reaction any more than is the deposition of calcium phosphate in this tissue. Fairhall and Shaw (1924) showed that under conditions of slightly increased acidity, tri-lead phosphate is readily converted into the much more soluble di-lead salt. The close analogy between the distribution, storage, mobilization and excretion of calcium and of lead in the organism has been clearly demonstrated in extensive studies by Aub and his co-workers (Aub et al., 1926; Hunter and Aub, 1927;

Bauer, Aub and Albright, 1929; Aub, Robb and Rossmeisl, 1932). Their findings have also been confirmed by many others, most beautifully perhaps by Behrens and Baumann (1933) in their work with radioactive lead. Factors which favor the deposition and storage of calcium in the skeleton also favor the storage of lead, while the same conditions which tend to dissolve calcium from the bones and bring it into the blood stream serve to bring about the solution and redistribution of stored lead. Thus the state of calcium nutrition, the acid base equilibrium, parathyroid activity, etc., all play a rôle in determining whether lead retained in the body is harmlessly stored in the bones or is allowed to circulate in the blood stream as a menace to the more vulnerable tissues of the body. The participation of so many variable factors obviously makes it impossible to predict the danger to an individual of a given exposure to lead.

Excretion of lead as a criterion of lead hazard. The amount of lead excreted, especially that appearing in the urine, furnishes a somewhat better criterion. Fecal lead is a mixture of unknown proportions of lead which has never been adsorbed and that which has been excreted by way of the intestinal tract. Urinary lead must have been absorbed and hence is of more significance. Kehoe, Thamann and Cholak (1933e) conclude, after an extensive study of persons industrially exposed to lead, that exposures are probably safe (i.e., will not cause clinical plumbism) if they do not cause a mean excretion of more than 0.6 mgm. of lead per day in the feces or more than 0.15 mgm. in the urine. The same authors found that lead poisoning could be expected in persons with a mean daily excretion of more than 1.10 mgm. in the stools or more than 0.21 mgm. in the urine. Similarly, Litzner and Weyrauch (1933) conclude that the urinary output of lead must be significantly higher than 0.10 mgm. per day before the assumption is made that the blood and tissues contain unusual amounts of lead. Meyers, Gustafson and Thorne (1935) place their estimate somewhat lower and believe that significance should be attached to the consistent presence of more than 50 micromilligrams (0.050 mgm.) of lead per liter of urine. All of these estimates of the amounts of urinary lead which should serve as a warning of possible danger of clinical plumbism are considerably higher than the amounts reported in the urine of persons without unusual exposure to lead.

Lead in blood as a criterion of hazard. Since the really important point is the concentration of lead to which the more vulnerable tissues of the body are exposed, a knowledge of the concentration of lead which

a person's exposure maintains in his blood would be the most useful criterion of the danger involved Such data involve the measurement of extremely small amounts of lead in a volume of blood which it is practical to withdraw from large numbers of people for chemical analysis. Whatever available method is adopted, gross errors are probably often introduced when slight traces of lead are detected and measured in 10 to 20 cc. of blood and the final result then calculated in milligrams of lead in 100 or 1000 cc. One should be conservative in accepting the figures now available as an accurate expression of the actual concen-

Lead found in blood of persons with varying exposures to lead

AUTHOR	METHOD USED	DEGREE OF EXPOSURE	LEAD IN BLOOD
			mgm. per 100 cc.
Kehoe, Thamann and Cholak (1933a).	Diphenyl carbazide	Modern American life	0.0 to 0.02
Kehoe, Thamann and Cholak (1933f).	Diphenyl carbazide	Significant industrial exposure	0.04 to 0.05
Kehoe, Thamann and Cholak (1933f).	Diphenyl carbazide	Patients with lead poisoning	0.09 to 0.36
Litzner and Weyrauch (1933).....	Adaptation of electrolytic procedure	Normal life	0.01 to 0.02
Litzner and Weyrauch.....	Adaptation of electrolytic procedure	Early symptoms of poisoning	0.04
Litzner and Weyrauch.....	Adaptation of electrolytic procedure	Unmistakable symptoms	0.06
Litzner and Weyrauch.....	Adaptation of electrolytic procedure	Frank lead poisoning	0.07 to 0.10
Tompsett and Anderson (1935).....	Dithizone	Hospital patients—no unusual exposure to lead	0.04 to 0.07
Tompsett and Anderson.....	Dithizone	Case of lead poisoning	0.380
Wexler and Sobel (1935).....	Spectrographic	Normal life	Definitely less than 0.10
Wexler and Sobel.....	Spectrographic	Clinical plumbism	0.10 to 1.0
Myers, Gustafson and Thorne (1935).	?	Normal in vicinity of New York City	0.006
Myers, Gustafson and Thorne.....	?	Subchronic intoxication	0.024

trations of lead present in the blood. Despite these reservations, a comparison of the concentrations found by the same investigators by the same chemical procedure in the blood of normal persons, as compared to the amounts detected in the blood of those industrially exposed to lead or of those with recognizable symptoms of plumbism, serves as a useful guide in evaluating the danger of the usual exposure to lead. Such figures are presented in tabular form.

While there is considerable lack of agreement in the concentrations of lead found in normal blood, where sufficiently complete data are

available, there is a reassuring difference in the amounts found in the blood of normal persons and in those with early signs of lead poisoning. More than this cannot be said until the actual amounts of lead involved are more definitely fixed by extensive studies in which small aliquots of the same sample of blood are analyzed by several different methods and these results compared with the analysis of much larger amounts of the same material.

Conclusions as to danger of poisoning from present average exposure. One can conclude from the data available that the daily lead intake of the average individual at present is considerably less than the intake which has been generally found to be dangerous. This exposure also results in a lower concentration of lead in the blood and less excretion in the urine than is usually associated with early signs of plumbism. These conclusions must be taken, however, as applicable only to the approximate period in which the studies were made. Only time can tell whether a continuation of modern industrial and agricultural practices involving the use of lead are going to increase gradually the magnitude of the lead exposure of the average individual.

PHYSIOLOGICAL ACTION OF SMALL AMOUNTS OF LEAD. The clinical signs and symptoms, as well as the pathological lesions which have been observed in chronic lead poisoning are too varied, inconstant and non-specific to furnish even a working hypothesis as to the fundamental actions of lead responsible for the deleterious effects produced in the organism. There is, therefore, even less chance that a study of these superficial manifestations will give any clue to the possible insidious harmful effects of amounts of lead too small to cause outspoken plumbism. A more useful approach is the study of the chemical and physiological actions of lead on simpler forms of living material and on those systems involved in the more constant signs and symptoms of chronic lead poisoning.

Effect of lead on growth of plant and animal tissues. Studies of the action of lead on fundamental biological processes are still rather fragmentary but suggest that further work may yield information of fundamental importance. Plant as well as animal growth is retarded by the addition of small amounts of lead to the nutrient medium (Bell, 1924; Dilling, 1926; Hammett, 1928; Hammett and Wallace, 1928). Concentrations of lead nitrate as low as 10^{-5} to 5×10^{-4} were shown by Hammett (1928) to have a definite slowing effect on the rate of root growth. The degree of retardation increased with increasing concentrations of lead. Hammett (1929a) showed conclusively that a hin-

drance of cell proliferation was the basis of the retardation of growth in length of seedlings. He was able to show that mitotic nuclei have the greatest affinity for lead, although the metal was demonstrated in both the nuclei and cell walls of roots (Hammett, 1929b; Hammett and Justice, 1929). Hammett (1929c) described tests which indicate that lead enters into combination with an organic sulphydryl compound analogous to if not glutathione itself. Lead was shown to retard the growth of chick embryos and here again the most pronounced effect was in areas of intense growth and rapid cell division (Hammett and Wallace, 1928). Dilling (1926) showed that lead concentrations higher than $\frac{N}{20,000}$ generally inhibited germination of frog spawn, while lower concentrations greatly lowered the percentage of eggs which germinated. Tadpoles which did develop were 40 per cent behind controls in size at the end of a month. Similarly plaice embryos living in sea water with 1 part in 250,000 of added lead showed 20 per cent retardation in growth as compared to controls at the end of six months. Because of the marked effect of lead on rapidly proliferating tissue which is rich in lecithin, Bell (1922, 1924) advanced the theory without convincing chemical evidence that lead combines with lecithin. High hopes were raised for a time that because of the predilection of lead for embryonic and rapidly growing tissues it would be of great value in the treatment of cancer (Bell, 1922, 1924). Dramatic results have been reported in some instances (Bell, 1926). However, further experience has shown little or no specific affinity of malignant tissue for lead.

Effect on enzyme activity. There is some evidence that lead interferes with normal processes in living tissue through its action on enzymes. Preti (1908) found that small amounts of lead hastened and that higher concentrations hindered the rate of formation of certain nitrogenous cleavage products in autolyzing liver brei. An increase in urinary nitrogen has been noted in animals following the administration of non-toxic doses of lead and in persons with lead poisoning (Preti, 1909; Tscherkess, 1925). These changes were interpreted as evidence of the action of lead on intracellular enzymes. Corran and Lewis (1928) report an increase in lipolytic activity in serum after the administration of lead. Recently with the use of more exact methods Dolowitz, Fazekas and Himwich (1937) have studied the effect of lead on oxidation, dehydrogenation and glycolysis in some of the tissues more commonly affected in lead poisoning. Oxidation studies were carried out in the Warburg respirometer, while dehydrogenation and glycolysis

were measured in Thunberg tubes. Brain, kidney, liver and testis were studied. Very small amounts of lead caused a marked decrease in oxygen consumption in all four tissues. Both increased and decreased respiratory quotients were observed in different experiments. In brain tissue lead caused an almost complete abolition of hydrogen transfer and a decided inhibition of glycolysis. These authors believe that through its action on enzymes lead causes profound metabolic changes in tissues which may be the underlying cause of varied manifestations of lead poisoning.

Effect on formation of immune bodies. The influence of lead on the rate of formation of immune bodies is another indication that lead profoundly influences the natural response of body tissues as a whole. When rabbits were given sufficient lead to cause poisoning (Bickert, 1929a, b, 1930 and 1931) the production of hemolysins and agglutinins was markedly increased, while the formation of precipitins was retarded. The formation of antitoxin against diphtheria was more rapid in animals receiving lead than in controls. It must be noted, however, that sufficient lead to cause poisoning was administered in these experiments and no conclusions can be drawn from these observations as to the effect of incidental small amounts of lead on immunity.

Effect on red blood cells. The common occurrence of anemia accompanied by the presence of stippled cells in the blood has made the red cell a favorite tissue in which to study the action of lead. The circulating red cells show rather marked morphological changes in lead anemia. Mayers (1926) has listed these changes in the order of frequency with which he observed them in 381 lead workers without acute symptoms of poisoning:

(1) Anemic red cells	71 per cent
(2) Anisocytosis	44 per cent
(3) Polychromatophilia	40 per cent
(4) Stippling	39 per cent
(5) Poikilocytosis	23 per cent
(6) Nucleated red cells	8 per cent

So-called "stippling" which is so valuable a diagnostic sign in lead anemia is due to the presence in red cells of discrete basophilic granules. The change is not an immediate specific effect of lead on red blood cells, since it is occasionally seen in other types of anemia and cannot be produced by the exposure of red cells to lead in vitro. Stippling does not occur in the blood of all species following the administration of lead.

For example, punctate basophilia is not observed in cats with lead poisoning but is a fairly constant feature of the intoxication in man, rabbits and guinea pigs. There is not complete agreement as to whether stippling is an evidence of degeneration or regeneration of erythrocytes. The change is most readily produced in young cells. Key (1924), who investigated the problem extensively, concluded that a stippled cell is an altered young red cell undergoing degenerative changes which need not necessarily be due to lead but which may occur when young red cells are exposed to lead in the circulating blood. Key was unable to find stippled cells in the bone marrow even when they were plentiful in circulating blood. Young and Osgood (1935), on the contrary, observed stippled cells in the bone marrow of animals poisoned with lead and regarded their presence as evidence of blood regeneration. Similarly Sellars (1921) interprets stippling as a result of regeneration under somewhat abnormal conditions.

The development of severe anemia accompanied by a high reticulocyte count indicates that lead in some way increases the destruction rather than hinders the formation of red cells. Studies of the marrow by Speransky and Sklianskaja (1928) support this view as they find a marked increase in erythroblasts in marrow counts in lead poisoning.

Considerable work has been done with regard to the nature of the injury due to lead. Aub, Reznikoff and Smith (1924a, 1924b) demonstrated that when 1 cc. of washed red cells are exposed to 0.01 mgm. of lead in the form of chloride they undergo definite and consistent changes. The cells decrease in volume, become much more resistant to hemolysis in hypotonic saline, lose their normal stickiness and at the same time become more subject to destruction by trauma. In other words, the cells lose their ability to swell and are more rigid and brittle and less resilient and durable. Ørskov (1935) found that the shrinkage in volume of red blood cells exposed to lead is accompanied by a rapid loss of potassium into the surrounding medium. Bicarbonate must be present in at least a concentration of 0.007 N in order for this action to take place and the optimum pH is from 6.3 to 6.6. Under these conditions lead in as low a concentration as 1:25,000,000 causes a marked increase in the permeability of red cells to potassium. In some experiments the cells had lost 40 per cent of their volume and 80 to 90 per cent of their total potassium content within 10 minutes. After these changes the resistance of the cells to hypotonic saline is greatly increased. The same reactions apparently occur in vivo, since Henriques and Ørskov (1936) found a decrease in volume and potassium

content of red cells in rabbits following the administration of lead. These phenomena cannot, however, be readily demonstrated in the blood of all species. For example, they appear in the blood of man and rabbits but not in the blood of cats and goats. The close parallelism between the species in which the effect of lead on red cells can be demonstrated and those in which anemia and stippling are prominent signs in lead poisoning points to the fundamental importance of these changes in the injurious action of lead.

When red cells are exposed to lead which has been previously mixed with serum the effects described by Aub, Reznikoff and Smith and by Ørskov are no longer produced. It has been shown that 0.01 mgm. of lead is completely inactivated by admixture with 0.2 cc. of serum. The inactivating substance in serum has been shown to be inorganic phosphate (Aub and Reznikoff, 1924; Ørskov, 1935). At first thought it would appear that in vivo red cells would be protected by the phosphate of plasma from the action of any amount of lead likely to be in the blood stream. However, it has been shown that lead affects red cells in living animals. Lead introduced into the blood stream comes in contact simultaneously with cells and plasma and reacts with both. Thus, although fewer cells would be injured by the same amount of lead in the presence of plasma, some cells would be affected, however small the quantity of lead present.

According to Aub and Reznikoff, lead probably combines with the inorganic phosphate of both serum and cells as follows:

$$2PbCl_2 + 2B_2HPO_4 \rightarrow Pb_3(PO_4)_2 + 4BCl + 2HCl$$

Lead would thus be transformed to the very insoluble and inactive tertiary phosphate with the liberation of free acid. They postulate that the injury to red cells is due to the locally increased acidity resulting from a collection of acid at the cell membrane. Maxwell and Bischoff (1929) disagree with this interpretation, since they could induce similar changes in red cells with lead compounds which did not cause the liberation of free acid. They believe that lead diglycerophosphate as well as the inorganic tertiary phosphate is formed when lead is brought into contact with erythrocytes. The diglycerophosphate is a much more active compound than the inorganic phosphate and its formation may account for the injury to red cells.

Whatever the mechanism is, exposure to lead serves to shorten the life of erythrocytes in circulating blood. The more rigid brittle cells are less able to stand the wear and tear of circulation and are destroyed

more quickly than are normal cells. Key (1924) believes that they disintegrate more by fragmentation than by hemolysis, as he found debris and hemoglobin-containing fragments in the livers and spleens of poisoned animals. The destruction of cells in the peripheral blood stimulates the marrow to form new cells. When regeneration is sufficiently rapid young forms appear in the circulating blood. The simultaneous occurrence of cells injured by lead and young cells poured out to replace those which are lost accounts for the blood picture seen in lead poisoning.

Effect on white blood cells. The white cells have received much less attention than the erythrocytes in studies of lead poisoning. Fine (1923) demonstrated that the phagocytic power of leucocytes is greatly reduced by their exposure in vitro to lead chloride in the concentration of 0.02 mgm. of lead per cubic centimeter. The mechanism by which this effect is produced has not been explained. Clinically the total white count is usually within normal limits in lead poisoning. Ferguson and Ferguson (1934) and Shiels (1936) have stressed the value of the lymphocytic ratio as a prognostic sign in persons exposed to lead. An increase in the ratio of $\frac{\text{large lymphocytes} + \text{monocytes}}{\text{small lymphocytes}}$ occurs early in exposure and later falls markedly as symptoms of poisoning develop. The authors feel that a gradual fall in this ratio in the blood of persons constantly absorbing lead is a valuable warning sign of impending danger.

Effect on skeletal muscle. It is well known that the function of skeletal muscles is impaired in lead poisoning. Using isolated nerve muscle preparations from frogs, Reznikoff and Aub (1927) showed that fatigue is more rapid and complete and recovery slower in "leaded" than in normal muscles. Their work indicates that interference is with muscle function rather than with nerve conduction. Fatigued muscles were shown to be much more susceptible than resting muscles to the action of lead—a difference which the authors attribute to the greater lactic acid content of fatigued muscle which increases the solubility and hence the reactivity of lead. An increase in the permeability of "leaded muscles" was indicated by the more rapid diffusion of inorganic phosphate into the surrounding Ringer solution. In view of Ørskov's findings in red blood cells, it would be interesting to know whether muscles also show an increased permeability to and subsequent loss of potassium. To our knowledge, no such studies have been made. Since potassium plays so significant a rôle in normal muscle function, a loss of this elec-

trolyte from muscle cells might be of tremendous significance in the impaired function following exposure to lead. Very recent work by Steiman (1938) shows a lowered level of phosphocreatine in the resting muscles of frogs exposed to lead. Following contraction in such muscles there is a marked interference with the resynthesis of phosphocreatine. It appears possible that there may be some correlation between the abnormalities reported by Steiman and the loss of inorganic phosphate reported by Reznikoff and Aub. At any rate, interference with the normal metabolism of so important a constituent as phosphocreatine must be an important factor in the ready fatigability, weakness and palsy seen in muscles as a result of exposure to lead.

Effect on smooth muscle. The frequent occurrence of colic and constipation in persons with chronic plumbism has turned the attention of many investigators to the action of lead on smooth muscles. It has long been a moot question whether smooth muscle is affected directly or through peripheral or central nervous mechanisms or by means of a combination of these factors. Aub, Fairhall, Minot and Reznikoff (1926) have critically reviewed much of the older controversial literature on the subject which need not be repeated here. In clinical lead colic or in the condition produced in animals by the injection of lead, increased tone and hypermotility of the intestinal tract can be demonstrated (Wassermann, 1916; Aub, Fairhall, Minot and Reznikoff, 1926; Grünberg, 1928). On the other hand, when intestinal strips are exposed to lead in vitro, the effect most consistently noted is inhibition of contractions usually with a simultaneous increase in tone (Smith, 1926; Dilling, 1926; Grünberg, 1928). In strips containing nerve elements Dilling showed that the effect of lead persisted after atropine and after nicotine and so concluded it could be of neither vagal nor ganglionic origin. He found that strips of uterine muscle behaved similarly to intestinal muscle when exposed to lead in vitro. Both Smith and Dilling were able to demonstrate this response with nerve-free rings of circular intestinal muscle and concluded that lead must affect smooth muscle directly.

Somewhat at variance with the observations of these authors are the carefully controlled experiments of Grünberg (1928) which definitely appear to implicate the autonomic system in the effects produced by lead, at least under the conditions of his experiments. He first studied the effect in vitro of lead on various types of smooth muscle. He included in this study smooth muscle from the intestines of various species, pregnant and non-pregnant uteri, etc. He found everywhere a

close parallelism between the action of lead, the action of adrenalin and the effect of sympathetic stimulation of the tissue concerned. In those tissues in which sympathetic stimulation caused contraction, lead caused contraction; while in those tissues to which the sympathetic is inhibitory, lead also caused a cessation of contractions. Lead injected into the mesenteric artery had the same effect on intestinal muscle as when applied locally in vitro. When injected in this manner lead is carried to the intestinal tract directly and is prevented by the liver from reaching the general circulation. Small doses of lead injected into the portal vein had no effect on intestinal movements. On the other hand, lead introduced into the general circulation always caused intestinal contractions. This effect persisted even when the blood supply to the intestines was cut off by ligation of the aorta at the diaphragm, but failed to occur if the vagi were sectioned in the neck. From these and numerous additional experiments, the author concluded that lead has two effects on intestinal activity: 1, it acts locally to inhibit movements by the stimulation of sympathetic endings; and 2, it acts centrally to cause intestinal contractions by stimulation of the vagus nerve.

Aub et al., Dilling and Grünberg all agree that the constipation which is so common in chronic plumbism is due to the inhibition of intestinal movements by the local action of lead. Aub attributes the griping pain of colic to a reflex constriction which approaches a non-motile hypertonic area, while Grünberg interprets colic as increased contractions due to the stimulation of the vagus center by lead.

Vascular smooth muscle apparently shares the common effect of lead on this tissue. The action has been demonstrated in vitro (Siccardi and Dozzi, 1914), but it is still a matter of discussion as to how significant a rôle the effect of lead on vascular muscle plays in either the functional disturbances or pathological lesions of lead poisoning. Certain investigators (Goadby and Goodbody, 1909; Legge and Goadby, 1912) have contended that the most specific lesion of lead poisoning is vascular damage resulting in hemorrhage. Others (Orphüls, 1915) have failed to produce vascular lesions in experimental poisoning. The pallor of saturnism, which is frequently out of all proportion to the degree of anemia, is often attributed to the constriction of the blood vessels of the skin. Hypertension is frequently present in persons with chronic lead poisoning and is thought by some to be caused by the action of lead on the vascular system. High blood pressure is also common in adults without lead poisoning, so it is difficult to know in a given case

whether lead is a causal factor. Only extensive statistical studies can give significant data on this point and the results of even such studies are somewhat conflicting. Vigdortchik (1935) studied blood pressure in 1437 lead workers and in 1332 persons not industrially exposed to lead. When the mass of data was statistically analyzed according to age groups, length of exposure, etc., the author came to the conclusion that prolonged exposure to lead increases blood pressure. This view found support both in the percentage of persons showing moderate or marked hypertension and in the average level of blood pressure in each age group of lead workers as compared to those not exposed to lead. Belknap (1936), on the other hand, in a careful analysis of data obtained over a period of years in 81 men heavily exposed to lead, concluded that there was no significant difference in the blood pressure of these workers and of normals of the same age groups. Petroff (1930) found hypertension during the early stages of experimental poisoning in animals followed later by normal or low blood pressure. During the period of hypertension he also observed tachycardia, hyperglycemia and increased cholesterol in the blood—all of which changes are compatible with increased sympathetic activity. He believed that much of the conflicting data with regard to the effect of lead on blood pressure in man may be attributed to the fact that various stages of the intoxication have been indiscriminately studied. Teleky (1937), after an extensive review of the literature, came to the conclusion that it has not yet been scientifically demonstrated that high blood pressure accompanies the signs of early lead poisoning in man, but emphasizes the need for further studies of the effect of prolonged absorption of small amounts of lead.

Judging from the similar response of all types of smooth muscle in vitro, it appears reasonable to expect that vascular tone would be affected, provided that a sufficient concentration of lead was present in the blood stream. This would be equally true whether lead acts directly on vascular muscle or through stimulation of sympathetic endings.

No studies are yet available which are concerned with the chemical reactions of lead with smooth muscle. It is interesting to speculate whether an increased permeability to certain inorganic ions with a subsequent change in electrolyte equilibrium may occur in smooth muscle as well as in red cells and skeletal muscle. Such changes, if present, might well be an essential factor in the functional disturbance of smooth muscle in view of the marked influence which inorganic cat-

ions are known to have on both smooth muscle and autonomic nerve activity (Howell, 1905; Zondek, 1922).

Effect on nervous system. Pathological lesions have been demonstrated in the brain, cord, meninges, autonomic ganglia and peripheral nerve fibers of individuals with lead poisoning. The presence of lead in brain, cord, and spinal fluid leaves little doubt that it is responsible for the abnormalities seen. Objective manifestations which indicate impaired function of the nervous system range from sluggishness, poor memory and irritability, to paralyses and the violent convulsive seizures of lead encephalitis. Aub, Fairhall, Minot and Reznikoff (1926) have given a critical review of the older literature and for more recent studies the reader is referred to Lehmann, Spatz and Wisbaum-Neuberger (1926), McKhann (1932), Villeverde (1933), Grünberg (1930) and Blackman (1937).

Outspoken pathological changes or gross functional disturbances of the nervous system are, however, manifestations of severe lead poisoning and a detailed description of them would add little to the present discussion. Here we must look for more subtle manifestations of impaired function and for actions of lead which may influence the normal physiology of nervous tissue. McDonald (1930), Rawkins (1931) and Porritt (1931) report such symptoms as apathy or irritability, lack of confidence, unfounded fears and inability to concentrate in persons with long exposure to small amounts of lead and without the more common signs of plumbism. They report a return to normal following removal of the source of lead. The possible causes of symptoms of this nature are so numerous and varied that a very extensive study would be necessary in order to establish any general causal relationship between their appearance and the absorption of lead.

The pathology in outspoken lead encephalopathy, according to many investigators, gives evidence of severe circulatory disturbance and vascular injury. Such observations may indicate that the prolonged absorption of smaller amounts of lead might result in less marked but still significant abnormalities in the blood supply to the brain. Dolowitz, Fazekas and Himwich (1937) have already been quoted as reporting a reduction in oxygen consumption as well as qualitative changes in metabolism in sections of brain tissue exposed to small amounts of lead. Since nervous tissue is extremely susceptible to oxygen lack, it is reasonable to suppose that impaired function might result from continuous slight interference with normal oxygen supply or utilization. There is also again the possibility that lead may cause

permeability changes in nerve cells analogous to those demonstrated in other tissues. Such an effect could hardly fail to impair the optimal functioning of highly specialized nervous tissue.

Effect on various other organs. Other organs in which changes in morphology or in function have been noted in outspoken plumbism and in which the insidious effects of small amounts of lead might affect the well-being of the individual, include the kidney, liver, sex glands and other glands of internal secretion. Much more will have to be known, however, about the exact nature of the action of lead in various tissues before slight abnormalities can with any degree of surety be attributed to the action of lead.

Possibility of acquired tolerance. The usual concept is that, while there is a wide variation in susceptibility, there is no true acquired tolerance to the injurious effects of lead. Legge and Goadby (1912), however, make the observation that individuals exposed to lead in industry for long periods appear to acquire a certain degree of immunity. Persons who develop signs of poisoning early in industrial exposure in some instances can continue in the same work without further difficulty. Weller (1926) in experimental studies in guinea pigs demonstrated the production of increased tolerance to lead. He first established the amount of lead which would produce meningo-cerebral symptoms in 100 per cent of experimental subjects. The survivors of the first attack were then given a rest period and later a larger dose of lead. By repeating this procedure he brought some animals to the point of tolerating four times the original dose without the production of symptoms. More experiments with smaller amounts of lead in various species are needed before any general conclusions can be drawn. Observations of this type, however, suggest that continued exposure may decrease the danger of small amounts of lead.

Need for new criteria for recognition of slight early injury from lead. From the entire foregoing discussion it is apparent that the recognition of the harm done by the small amounts of lead absorbed by the average individual is bound to be difficult. The investigations which give evidence of the marked influence of lead in great dilution on various isolated tissues incline one to the view that the continuous absorption of any amount of lead must result in less than optimal conditions for the organism as a whole. At present, however, we do not know what to look for in the average individual as subjective or objective manifestations of this slightly unfavorable condition. From the available

estimates of the present lead exposure and the amount absorbed by the average individual, it appears doubtful that the usual recognized signs and symptoms of plumbism are to be expected in more than an occasional highly susceptible person. There may conceivably be many instances in which lead is contributing to constipation, or to a tendency to anemia, to ready fatigability of muscles, to hypertension or to impaired nervous and mental function. There are, however, many other possible causes of exactly the same abnormalities. Data on the amount of lead present in the blood and urine of persons both with and without symptoms of this type may eventually make possible a correlation between their appearance and the absorption of lead. To be useful for this purpose the chemical data must be accurate and the studies must include statistically significant numbers of individuals both with and without any recognizable deviation from normal.

PRESENT MOST URGENT PROBLEMS. Probably the question of greatest concern at present is whether modern practices are going to result in a gradually increasing lead exposure for the average individual. Again, it appears that the most satisfactory answer to this question is to be afforded by studies, repeated at intervals, of the lead in the blood and urine of large and representative groups of persons in both rural and urban communities. There is no doubt, as was stated at the beginning of this paper, that the continued absorption of amounts of lead which are still relatively small result in outspoken poisoning. It is important, therefore: 1, to keep informed as time goes on as to the margin of safety which we have between the lead exposure of the average individual and those amounts generally recognized as dangerous; and 2, to establish a more delicate set of criteria than we have at present for the recognition of early slight injury from lead.

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FACTORS CONCERNED IN THE DURATION OF PREGNANCY

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In the attempt to account for the emptying of the uterus at a definite time, various hypotheses have been formulated upon the basis of one or more of the changes in the fetal or maternal organism which have been noted at the time of termination of pregnancy. The viewpoint of the obstetrician, whose experience involves chiefly the phenomena of labor, has been the basis of numerous theories, the multiplicity of which bears evidence of the uncertainty attending them.

A clue to the nature of parturition is found by experiment. Emptying of the uterus is under hormonal control; the time of onset may be hastened or postponed as desired, living offspring being born in either case. In experiments in which a new ovulation is superimposed upon pregnancy, a definite rhythm is still evident in the duration of gestation; the amount of lengthening or shortening of pregnancy is not irregular, but is fixed according to the length of the ovulation cycle; the normal mechanism of labor seems to be in operation except at an earlier or later time than is usual.

In order to determine the relation of parturition to preceding stages of pregnancy, the method of the embryologist rather than that of the obstetrician must be followed. The common method of approach to a problem of embryology, whether a structure or a function is involved, is to reconstruct various stages in the development of the organ or process. The final form is viewed from the standpoint of its development.

From the embryological standpoint, parturition represents merely the terminal phase of a long series of events constituting embryonic life. The brief duration of the period of emptying of the uterus (a few minutes in rodents, a few hours in the human), in contrast to the long period of retention of the fetus, has been a major obstacle, making it difficult to detect the factors which control the process. The question as to how far back in embryonic life one must seek for the origin of the birth mechanism involves investigation of the rôle of various endocrine organs at different stages of pregnancy. The minimum requirement of en-

docrine function in various species may be revealed by determining whether pregnancy is maintained or interrupted following extirpation of various organs of the endocrine system. From this point of view, knowledge of the control of parturition is not so much a question concerning the mechanism of expulsion of the contents of a distended muscular organ, involving only a few hours, as it is a matter of determining what the factors are which cause retention of the fetus throughout the long period of gestation.

The results of such inquiry reveal much evidence which shows that not only parturition but also other critical phases of embryonic life, e.g., ovulation, fertilization, transport of the ova, implantation, are under hormonal control, and by this means are coördinated in a sequence with a fixed time relationship which is constant for a given species. Thus a delay of implantation for several weeks regularly results in postponement of the attainment of mature development of the fetus and of parturition, for a period of equal duration. The rate at which changes occur in the generative organs at the beginning of pregnancy is the basis upon which the termination of gestation is coördinated. The question as to why the embryo should suddenly be cast forth after remaining so long within the uterus falls into the same category as the question as to why the blastocyst should remain unattached to the uterine wall for so long a time after escape from the tube.

A chief source of confusion as to the physiological mechanism of parturition has been obscurity regarding the means of coördination of various phases of pregnancy. Direct evidence of the timing mechanism responsible for the rhythmicality of gestation is available, in so far as overt manifestations of the sexual cycle can be demonstrated to recur, rather than to be completely suppressed during pregnancy. By this means the periodicity of the cycle is linked directly with that of gestation; the actual length of pregnancy comes to approximate closely a simple multiple of the cycle.

The present point of view regarding this problem is arrived at by taking into account the following evidence: first, that the duration of pregnancy is under hormonal control, as illustrated by experiments in which the duration of gestation is lengthened or shortened as desired: second, that the endocrine requirement which is minimal for the successful maintenance of pregnancy of normal duration in various species, is likewise closely related to that which is essential for a cycle of normal duration: third, that pregnancy is composed of a series of cycles which are not completely suppressed, the cycles being the functional units or

links in the chain of events constituting pregnancy, by which a timing mechanism is set at a given rate for a species.

I. INHIBITION OF PARTURITION. Experimental inhibition of the entire process of parturition affords an especially interesting starting point from which to view the problem (76, 40, 45, 44, 43, 42, 5, 21, 56, 50, 29, 39). For example, in rabbits in which ovulation is induced at the twenty-fifth day of pregnancy, by the injection of a single dose of extract of urine of pregnancy, parturition fails to occur at term (32 days). Inhibition of labor is linked with the presence of a new set of corpora lutea which have developed to the stage of maximal size and function by the thirty-second day, when gestation normally would terminate. Under these conditions pregnancy is prolonged, the onset of parturition being delayed until fifteen days after injection, i.e., the fortieth day, or until the end of the life span of the induced corpora lutea.

That the induced set of corpora lutea are functional is shown by the following tests: 1, Microscopical examination; the ovaries at term contain a fresh set of corpora lutea at the stage of full development. The uterine mucosa of the non-pregnant horn in unilateral pregnancy, shows changes characteristic of the presence of active corpora lutea. 2. Absence of ovulation after coitus; the period of inhibition of ovulation which normally ends at term, is prolonged a week past term. 3. Absence of pituitrin effect; pituitrin administered at term fails to induce parturition, despite a dosage 1000 times greater than the amount normally effective.

The duration of these changes, namely, the deferred onset of parturition, the prolonged period of inhibition of ovulation, and the failure of the full term uterus to respond to pituitrin correspond in general to the life span of the experimentally induced set of corpora lutea. When it is remembered that in the rabbit the corpus luteum is active for 14 to 16 days, even though pregnancy does not occur it is evident that the changes observed in these animals conform chronologically to the normal physiology of the corpus luteum.

These experiments aid in the evaluation of theories regarding the relation of changes in the fetus and in the placenta to the onset of parturition. When labor failed to occur it was found that the fetuses survived and continued to grow until the size, weight and conspicuous covering of hair corresponded to that of newborn rabbits three days after birth. In fact living fetuses were removed by hysterotomy thirty-five days after mating. The prolonged retention of the fetuses past term gave an unusual opportunity to observe the length of time during

which the placental function was adequate to support fetal life. Survival of fetuses about three days past term, or until the thirty-sixth day, indicated the functional reserve of the organ; and even after death of the fetuses it was found that the placenta remained implanted in the uterine wall, and glycogen still persisted in placentae extruded at forty-one days. It is evident that these experiments lend no support to the view that the onset of labor is caused by changes in the fetus (63, 26a), senility of the placenta (28, 17), or mechanical distention of the uterus (62, 31, 32).

INDUCTION OF PARTURITION. The induction of labor with birth of living fetuses, as well as the inhibition of it, is under hormonal control (76, 1, 24). Pregnancy may be shortened as well as lengthened in the rabbit by a single injection of extract of pregnancy urine, the reaction being determined by the dosage and by the stage of pregnancy. In fact, these factors may be adjusted so that in a given animal both types of response can be elicited; certain fetuses of a litter may be cast out prematurely and alive, while the rest of the litter survives in the uterus and eventually attains postmature stages of development.

When labor is induced by this method during the last quarter of pregnancy there are certain distinctive characteristics. The fetuses are cast out alive, or usually not macerated. Vaginal bleeding is often profuse after separation of the placentae. The onset of parturition occurs after a latent period of more than twenty-four hours, and the duration of expulsion of fetuses is limited to the second or third day following injection.

In summary, the duration of gestation may be controlled in the rabbit by the induction of ovulation. The change in the time of onset of parturition, whether postponing the termination of pregnancy, or hastening it, still follows a definite pattern. Emptying of the uterus occurs either at the beginning of the sixteen-day life span of the induced corpora lutea or at the end of it. Since living fetuses are born when pregnancy is interrupted by this method, the procedure offers the functional equivalent of spontaneous parturition. It is chiefly the duration of pregnancy which is altered. The normal mechanism of labor seems to be in operation except at an earlier or later time than is usual. The duration of pregnancy may be formulated in terms of hormonal function.

CHANGES IN THE UTERUS DURING PARTURITION. How the endocrine organs actually influence the gravid uterus to yield its contents may be clarified by direct examination of the uterus. Unusually favorable conditions are found in rabbits in which ovulation has been induced (76).

1. With regard to the state of contractility of the uterine muscle at the time of the emptying of the uterus of living offspring, it was found that birth of living fetuses readily occurred during the period of maximal quiescence of the uterine musculature, when the ovaries contained fully developed corpora lutea at the stage of maximal activity. For example, in rabbits injected with urine extract at twenty-five days, a part of the litter involving fetuses from one or both horns, may be cast out alive at twenty-seven or twenty-eight days, while the rest of the fetuses survive within the uterus until long past term, being expelled usually about the forty-first day of pregnancy. The survival of the fetuses which are implanted adjacent to those which are cast out alive, is direct evidence of the relatively quiescent state of the uterine muscle, although still functioning adequately for the expulsion of living offspring.

2. Dislodgment of certain fetuses of a litter is not caused by muscle contraction alone, but is related to structural changes of the decidua, involving the placenta. Various stages of partial separation of the placenta were found upon gross examination of a series of uteri obtained on the second or third day following induction of ovulation at twenty-five days. Antepartum vaginal hemorrhage is a striking feature of labor under these circumstances and excessive bleeding also marks the postpartum stage. Microscopical examination reveals edema and intense hyperemia of the uterine mucosa, associated at times with extravasation of blood involving the entire uterine wall. Induction of parturition is effected by means of induced premature separation of the placenta, rather than by a muscle response. The birth of living fetuses also suggests that hyperemia is the essential feature rather than anemia such as that associated with thrombosis or vascular changes of degenerative type, which have often been emphasized in studies upon placental infarction in relation to labor.

When one bears in mind that in all mammals the development of fresh corpora lutea is marked by hyperemia and growth of endometrium (79), it is not difficult to understand the nature of the changes in the decidua when a fresh set of corpora lutea is added during pregnancy. Since ovulation can be induced at any stage of pregnancy in the rabbit, a method is available by which the structure of the decidua can be altered at any time which is selected (77, 39). In fact, when put to the test, in a series of 200 animals during the last quarter of pregnancy, it was found that variation in the time of injection of the urine extract resulted in termination of pregnancy by abortion, premature labor, full term labor, or postmature labor, as desired, living fetuses being born at all stages (76, 76a).

In brief, when the process of parturition was slowed down so that the duration of the period from the onset of expulsion of the first fetus until extrusion of the last one of the litter was two weeks, the dissociation of the mechanism of labor was not entirely irregular, but occurred in two phases. The part of the litter which was not cast out during the first phase, i.e., on the second or third day after ovulation, was retained in the uterus and grew in size to the maximum which was possible in the intra-uterine environment; finally, the second period of expulsion intervened about two weeks after induction of ovulation. The two phases differ strikingly with regard to the changes which occur in the uterine wall, although both lead to emptying of the uterus. In the first period decidual changes predominate, the muscular contractility is minimal; in the second phase, muscular contractions are uninhibited, response to pituitrin has returned, decidual changes are slight.

Insofar as labor involves the uterus, two cardinal factors stand out, namely, the decidual and the muscular; evidence of their respective rôles in parturition is obtained by attempting to exhibit them one at a time, since normally they are not dissociated.

The relative prominence of the decidual or muscular changes may vary according to the stage at which pregnancy terminates. Dominance of the muscular factor is a striking feature of labor at term in the rabbit or human. In abortion at an early stage of pregnancy, however, the product of conception may escape with relatively little evidence of muscular activity accompanying detachment of the placenta.

PROLONGATION OF PREGNANCY BY DELAY OF IMPLANTATION. The duration of pregnancy may be influenced not merely by the inhibition of parturition at term, or by the induction of premature labor, but also by the postponement of implantation. Alteration of the period of gestation in this instance also, is under hormonal control.

Prolongation of pregnancy was noted by Lataste (49) in 5 species of rodents, occurring in animals which were suckling their young despite the beginning of another pregnancy. Since mating and ovulation may occur in rodents within a few hours after parturition, pregnancy begins early in the lactation period. Under these circumstances offspring of normal size are born; only the duration of pregnancy is abnormally lengthened. This result clearly shows that development of the embryo may be retarded or completely arrested in the course of pregnancy.

Apart from the influence of lactation, the existence of a period lasting 2 to 6 months during intra-uterine life when little or no development of the embryo occurs, has been established in a dozen species of mammals,

as Hamlett (30) has pointed out. Arrest of development occurs at a definite time, namely, during the blastocyst stage, which thus may persist for months. Finally implantation occurs, whereupon growth of the embryo is resumed with the same abruptness with which it left off, and at a normal rate as though no interruption had occurred (19). For example, in the nine-banded armadillo, in which the uterine contents were examined at various stages of pregnancy, Hamlett found that in the course of a gestation period of 8 months, there were blastocysts present during the first 4 months, while the entire development subsequent to implantation occurred during the last 4 months.

Numerous experiments show that delay of implantation, and the consequent discontinuity of development of the embryo, are related to the state of activity of the corpus luteum. Suckling prolongs the life span of the functional corpora lutea. In rats and mice, the suckling of a foster litter prolongs pseudopregnancy (69); the effect does not occur in ovariectomized animals (70). The delay in implantation in mice is related to the number of suckling young; the greater the number of suckling young, the later is implantation; each young suckling prolongs gestation about one day (20). Injection daily of anterior hypophyseal extract in rats, starting on the day of mating, delayed implantation, and prolonged pregnancy 2 to 6 days; the ovaries were much enlarged, due to increase of lutein tissue (80). Pincus injected progestin into lactating mice over a period of 5 to 8 days during early pregnancy and found that normal litters were born, but that the average date of birth was 4 days later than would occur in prolonged pregnancy due to lactation alone if the expected delay is calculated on the basis of 21 hours per suckling young (59).

In summary, two types of prolongation of pregnancy result from prolongation of activity of lutein tissue, namely, that due to inhibition of labor, and that resulting from delay of implantation. In the former a new ovulation cycle is superimposed upon normal pregnancy at a late stage; in the latter, nature has provided the essential conditions of experiment, namely, the close relation of the period of parturition with the period of estrus and ovulation. Whether the new ovulation precedes parturition, as in the first type, or follows it closely, as in the second type, the time pattern of pregnancy is altered, although not in totally irregular fashion. The termination of pregnancy still coincides with the termination of the experimentally induced endocrine activity. Parturition is influenced by the same hormonal factors which are required for implantation.

II. Basic factors in the hormonal control of pregnancy. In or-

der to determine the minimal requirement of hormonal function which is essential for the maintenance of pregnancy, one may consider first, the differences of endocrine control in various species, and second, the results of extirpation of the principal endocrine organs upon the retention of fetuses within the uterus.

Ovary. In certain species, e.g., opossum (34), rabbit (10), mouse (58), the presence of the corpus luteum is essential during the whole period of gestation; ablation promptly results in the interruption of pregnancy. On the other hand, in the horse and donkey mare, there is no corpus luteum in the ovaries during the latter part of pregnancy (9). Extirpation of the ovaries at 200 days in the mare did not affect the normal course of pregnancy; parturition occurred at 347 days, as usual; estrus failed to re-appear postpartum, thus confirming the completeness of castration (33).

In the guinea pig both ovaries may be removed as early as the end of the first trimester (23 days) of pregnancy without interruption of gestation (90, 38). Extirpation of the corpora lutea 6 days after copulation may not be followed by abortion immediately (52, 51). A new ovulation may occur in the pregnant animal, taking place about a week following removal of the corpora lutea. The animals were sacrificed 19 days after mating, the corpora lutea having been absent during a period of 6 to 9 days preceding the new ovulation. Since implantation occurs in the guinea pig about $6\frac{1}{2}$ days after mating, the foregoing observations indicate that after the uterus has reached the development characteristic of implantation, absence of corpora lutea during the first week following implantation does not necessarily result in the prompt interruption of pregnancy in this species.

In the cat, removal of both ovaries at 49 days or later, was followed by normal parturition at term (64 days), although castration at 46 days, or earlier, regularly interrupted pregnancy (27).

In the human, numerous instances of castration in early pregnancy have been reported, in which no interruption occurred (3); bilateral ovariectomy at 34 days may be followed by normal birth at term, and complete amenorrhea thereafter (82). In the rhesus monkey, under controlled conditions of experiment, Hartman has found that removal of the ovaries as early as the second month of pregnancy may not interrupt gestation (35).

The formation of a placenta is dependent upon the corpus luteum. Numerous experiments in many species have shown that the uterine response necessary for normal implantation or the production of deciduomata is linked with the development of the corpus luteum.

In the period preceding implantation, there is evidence that the corpora lutea have a decisive rôle in the nutrition and transport of the ova, and thereby influence the course of pregnancy. The passage of ova through the tubes, which normally requires 3 days in the rabbit, may be delayed by extirpation of the corpora lutea 15 to 20 hours after mating; in one animal degenerating blastocysts were found in the tubes $7\frac{1}{2}$ days after coitus (10). Acceleration of the rate of transit of the ova through the tubes occurs in pregnant or pseudopregnant rabbits in which a second ovulation has been experimentally induced; ova of the second or induced ovulation reach the uterus in less than $2\frac{1}{2}$ days following injection of extract of pregnancy urine (89).

The ova undergoing cleavage in the uterine cavity are not distributed in totally irregular fashion, but a coördinating mechanism operates to place them in approximately equal numbers in each of the communicating horns of a bicornuate uterus (11, 54).

Anterior pituitary. Extirpation of the hypophysis in the rabbit at any stage of pregnancy results in the interruption of gestation (24, 87, 85). In the later stages of pregnancy, the offspring may be cast out alive. On the other hand, in rats (60), mice (71) and guinea pigs (61), hypophysectomy during the latter part of pregnancy is not followed by premature emptying of the uterus, but living fetuses are retained until birth occurs at term or later. For instance, in rats hypophysectomized before implantation, e.g., on the 4th day of pregnancy, implantation fails to occur; in animals operated soon after implantation, e.g., on the 7th to 10th day, pregnancy fails to continue; operation at 11 to 20 days results in prolongation of gestation with the birth of living or dead fetuses several days past term; operation on the 21st day is followed by normal birth at term a day or two later.

The mechanism responsible for the interruption of pregnancy on the one hand, and the prolongation of it on the other, is revealed by examination of the ovaries. In the rabbit, degeneration of the corpora lutea promptly follows hypophysectomy (72); in contrast in the rat, no similar regressive changes are noted; on the contrary, persistence of the corpora lutea has been observed 15 months or longer in hypophysectomized rats (74).

The anterior hypophysis is concerned primarily with the starting of pregnancy. Ovulation depends upon it (23, 85); later stages in the development of the ovarian follicles are also under pituitary control (73). Growth of the ovum itself during early follicular development is independent of the hypophysis; in hypophysectomized animals, the

ova attain the full size of mature ova of ovulating animals, full growth of the ovum being reached in rats and mice by the time antrum formation begins (59).

It may be noted that the endocrine organs found by extirpation experiments to be essential for pregnancy, namely, the corpus luteum and anterior hypophysis, are the identical ones which are indispensable for the cycle.

Placenta. The endocrine function of the placenta is illustrated by the prolongation of the life span of the corpus luteum which follows implantation and growth of the chorion. The state of activity of the chorion influences the time of emptying of the uterus, as is shown by the course of pregnancy following death of the embryo. In the human, Streeter (78) has shown that the death of the fetus between the 6th and 17th week of pregnancy is not followed by emptying of the uterus at once, but as a rule the dead fetus and the placenta are retained about 6 weeks. It is well known that retention of the fetus at this stage of pregnancy is associated with refractoriness of the uterine muscle to pituitrin, in contrast to the striking effect at term. One may view the latent period of about 6 weeks as a measure of the interval which is required for changes in the placenta and retrogression of the corpus luteum to reach the stage at which the uterine muscle is released from the inhibitory influence which normally is operative throughout pregnancy, preventing escape of the fetus from the uterus.

In mice (57), in which death of the embryos at 12 to 15 days was produced by pressure without dislodgment of the placentae, delivery of the retained placentae did not occur until full term (21 days), and was followed by estrus in 1 to 2 days. In control animals, removal of fetuses and placentae at 12 to 15 days was followed by estrus in 3 or 4 days. Despite the degenerative changes in the placenta following death of the embryo, there is evidence of placental activity until term at a level which maintains the inhibitory effect of pregnancy upon the sexual cycle in species in which gestation is short (47, 46, 2, 86).

In view of the influence of the placenta upon the ovary in the early stages of pregnancy, a special rôle in the maintenance of pregnancy at later stages, too, has been suggested for the placenta. Especially in certain species in which removal of the ovaries may not interrupt pregnancy, e.g., horse, guinea pig, cat, monkey and human, it has been suspected that the placenta functions as an endocrine organ, compensating for ovarian deficiency. A function which the placenta has in common with the ovary is the production of estrogenic hormone. In fact, as

Corner has shown, it is highly probable that the ovary and the placenta are the only sites of formation of this substance in the animal body (12).

In the mare, the source of estrogenic substance has been traced to the fetal portion of the placenta (7). In this species estrin disappears abruptly from the urine of both mother and foal within a few hours after parturition. This is much more rapid than the rate of disappearance of estrin following delivery in the human, in which case a week or longer is required. In explanation of the difference of the postpartum period in the two species, it may be pointed out that fragments of chorionic tissue are known to be retained in the human, due to complications of placental separation inherent in a hemochorial type of attachment. On the other hand, in the mare detachment and elimination of the entire chorion at birth probably is greatly facilitated by the epithelio-chorial type of placentation.

Posterior pituitary. Extirpation of the pars nervosa of the hypophysis, leaving the stalk intact, was followed by normal birth at the usual time in rats (75). There is evidence, however, that the stalk must be taken into consideration in any attempt to determine the function of the neural division of the hypophysis. Gersh (26) has demonstrated the glandular nature of certain cells which are distributed throughout the neurohypophysis, including the stalk. Parturition in rats was accompanied by hypertrophy of these glandular cells; similarly dehydration resulted in their enlargement. Hypersecretion of the antidiuretic substance was indicated, and perhaps also oxytocic substance. It was not clear whether the cellular hypertrophy of the postpartum glands was entirely in response to the need for conservation of body water such as lactation might require, or whether it was, in part at least, a response to the excess secretion of oxytocic substance.

Direct evidence of a neuro-humoral factor in parturition was sought by Fisher, Magoun and Ranson (25) in 7 cats in which interruption of the hypothalamico-hypophyseal tracts was accomplished at various stages of pregnancy. Gestation was maintained, but in all animals emptying of the uterus at term was exceedingly sluggish, or even failed to occur; only one animal survived in good health. All showed diabetes insipidus and the hypophyses from three of the animals were shown to have very little antidiuretic activity, and problably would have proved to be devoid of oxytocic activity had they been tested. The present evidence does not warrant the rejection of pituitary oxytocin as a possible factor involved in normal parturition.

The possibility of an effect upon the anterior lobe following stalk section was suggested by the failure of operated cats to come into heat

or to become pregnant. In rabbits after severance of the pituitary stalk, Brooks (6) found that ovulation failed to occur following mating, although injection of extract of pregnancy urine did cause ovulation; thus the ovarian response resembled that characteristic of pregnancy. Histological evidence of interruption of nerves which pass down the stalk from the hypothalamus to the anterior lobe was obtained, since fibres were demonstrable in the anterior lobe before section of the stalk but not following it.

III. Relation of the cycle to pregnancy. Total suppression of cyclic changes throughout pregnancy does not occur, although considerable variation in this regard is evident among different species.

Ovary. Ovulation occurs spontaneously in the horse during pregnancy (9). A great increase in lutein tissue begins about the 45th day, thus occurring subsequent to implantation. Numerous freshly ruptured follicles augment the lutein tissue consisting of the single corpus luteum which dates from the time of mating. Furthermore, the blood first shows gonad stimulating hormone about this time (37th to 42nd day) (8). Disappearance of this substance from the blood (130th to 180th day) is marked by regression of the corpora lutea. In fact, no corpora lutea are to be found in the ovaries during the last trimester of pregnancy, the gestation period being about 11 months.

In the cow (31), during pregnancy, the graafian follicles may attain their full size, excluding the pre-ovulation increase; there is also a great increase in the number of atrophic follicles. Estrus and mating occasionally occur at almost all stages of pregnancy. Just before parturition, the vulva and vagina become red and swollen; the symptoms are very similar, but more intense than those which occur at the end of pseudopregnancy or during a normal heat period.

In the guinea pig, extirpation of the corpora lutea 6 days following coitus may result in a second ovulation during pregnancy (52). In the mouse, ovulation may be readily induced without interruption of pregnancy by implantation of a single hypophysis (91). In the rabbit, ovulation is readily induced throughout pregnancy by urine extract (77). The failure of ovulation after coitus during pregnancy seems to be related to interference with post-coital release of pituitary hormone (possibly a direct effect of corpus luteum activity upon the hypophysis), rather than lack of follicular sensitivity, since the ovarian follicles will ovulate in response to a minimal ovulating dose of gonadotropic hormone (53, 84). The functional state of the follicular mechanism may thus be tested during pregnancy, and the threshold of response determined.

In the rat, the rhythm of the follicular cycle is not obliterated during pregnancy (22). In this species, which has a gestation period of 4 or 5 times the length of the estrous cycle, 4 or 5 cycles of the production of eggs and follicles and their degeneration occur during the period. Beginning with the 5th day of pregnancy, a few small corpora may be found, in some of which the retained egg can be seen, although normal ovulation has not been observed at this time. Nelson observed an animal in which estrus recurred regularly throughout pregnancy, being accompanied by copulation and typical changes in the vaginal smears (55).

The guinea pig also shows a recurrence of the follicular cycle during pregnancy, the large follicles becoming atretic without luteinization. Signs of estrus during pregnancy have been noted occasionally (51). From the knowledge available in various species, including the ferret, cat, rhesus monkey and human, Evans and Swezy find no evidence that gestation inhibits the normal growth and maturing of follicles, though ovulation seems to be inhibited.

Uterus. In the human, menstruation at regular intervals during pregnancies of normal duration has been described by Wintz in 2 cases in which periodic bleeding occurred in the empty horn of a bicornuate uterus (88).

In various laboratory animals with unilateral pregnancy, e.g., the mouse and rabbit, the nonpregnant horn does not remain unchanged throughout pregnancy (15). As term approaches, the endometrium shows alterations, being accompanied in the mouse by hemorrhage in the stroma preceding parturition. In the rabbit, histological changes appear about the 18th day; the development characteristic of early pregnancy is not maintained (13). The structure of the endometrium of the nonpregnant horn of the rabbit may be altered by the induction of ovulation at the beginning of the last quarter of pregnancy, the changes paralleling the growth of the new corpora lutea. Fertilized ova, even at the blastocyst stage, failed to become implanted, however, in the presence of pregnancy in the opposite horn (16); likewise deciduomata failed to form (14).

Changes in the musculature at various stages of pregnancy may be demonstrated by the effect upon the course of pregnancy following administration of pituitrin (48), as well as by the difference in sensitivity of isolated strips of muscle obtained at different periods of gestation (64, 65).

In the rhesus monkey, Hartman (36) has shown that immediately following implantation the factors involved in the maintenance of the

sexual cycle still operate, though much dampened down, so that one, or at most two abortive cycles result. These cycles seldom attain the bleeding stage after the hemorrhage of implantation, i.e., the placental sign, has disappeared. In the human, Bartelmez (4) has pointed out, there are indications that a similar bleeding occurs. In a chimpanzee, Tinkelpaugh (81) noted the recurrence throughout pregnancy of the periodic genital swelling, nine periods being observed during the gestation period of approximately 9 lunar months. Elder and Yerkes (18) have also reported recurrence of genital swelling in 5 pregnancies, although periodicity was less regular.

The presence of certain components of the sexual cycle throughout pregnancy and the suppression of others brings up the question as to what relation exists between the various overt manifestations of the reproductive process, and whether or not various levels or thresholds of response exist. In the monkey, Hartman determined the sequence of events in both adolescent (pre-ovulation) animals and in mature ones, the latter being observed during all phases of reproductive activity, including pregnancy and lactation. He observed that the various signs of the sexual cycle may increase gradually in "staircase" fashion from one cycle to the next, finally to culminate in ovulation. From a state of amenorrhea, return of the cycle is usually gradual; coloration of the genital region and periodic bleeding return first, an increase of vaginal desquamation follows, while growth of the ovaries and uterus always occurs last. The threshold for certain phenomena, e.g., bleeding, vaginal desquamation, is far below that of other changes, such as ovulation. From Hartman's description of the nature of the sexual cycle one may understand more readily the different levels of cyclic activity of various species during pregnancy and also the variations among individuals of the same species.

In so far as there is evidence of the persistence of cyclic changes throughout gestation, the rhythm of the cycle is linked directly with that of pregnancy. It has been a problem to account for the fixed duration of pregnancy, which is dependent upon coördination of such precision throughout gestation that the time of termination can be foretold, if the beginning of the process is known. Evidence has been cited of a mechanism by which the actual length of pregnancy comes to approximate closely a simple multiple of the cycle. From this standpoint, pregnancy is composed of a series of cycles.

Concerning the time of onset of parturition with respect to the series of partially suppressed cycles, there is evidence that the time of termina-

tion of pregnancy is related to the time of termination of a sexual cycle. This seems to be the significance of the fact that, as a general rule, the length of pregnancy equals a simple multiple of the cycle.

In animals with a short gestation period, like the mouse (21 days) or rabbit (32 days), in which a maximum of coördination is required of the endocrine mechanism controlling retention of the fetuses within the uterus, there is a close relation between the end of gestation and the end of a cycle. The corpora lutea have been shown to be functional in these species until the terminal phase of pregnancy; decline in this activity is associated regularly with emptying of the uterus. The period of parturition is closely related to the period of estrus; fertile mating occurs on the day of delivery. The superimposing of a new cycle upon pregnancy in the rabbit by the induction of ovulation alters the duration of pregnancy not irregularly, but according to the rhythmic pattern characteristic of the sexual cycle of this species. The experiment demonstrates the dominance of the basic cyclic response in causing the termination of pregnancy. Just as the period of estrus can be predicted with confidence to occur in the cow or pig at an interval of 3 weeks from the time of the last one, and the onset of menstruation can be foretold in the human or macaque at a date 4 weeks from the previous menstrual period, likewise another striking external manifestation of the functioning of the reproductive system, namely, parturition, can be predicted to occur at a fixed period even a year in advance.

Variations in the length of the gestation period have been of considerable interest with regard to their bearing upon the changes occurring at parturition. Rosahn, Greene and Hu (66) made a statistical analysis of 569 pregnancies in rabbits of 11 standard breeds. The gestation period varied from 29 to 36 days, although the mean gestation period ranged from a low of 30.37 days for a Polish breed, to 32.89 days for the Albino group. It may be noted that at 29 days the period of viability begins, i.e. at 28 days newborn rabbits fail to survive (67). At 36 days the period of postmaturity ends, i.e., intra-uterine death occurs. The larger the series of cases, the more likely are these extremes to appear, as Schwarz (68) also found in similar investigations of other species. Since the mean duration of pregnancy in the 11 breeds averaged 31.45 days, it is evident that the extreme range of 29 to 36 days is of minor importance in a consideration of normal gestation. The constancy of the gestation period was emphasized by the finding that the variance between breeds was significantly greater than the variance within breeds. Between breeds prolonged gestation periods were associated with large litters and short gestation periods with small litters; the opposite was

true within a given breed. It was concluded that the differences in the gestation periods of different breeds of rabbits were dependent upon hereditary factors, since the environmental factors of housing, feeding, and season, and age of the doe could not account for the observed heterogeneity.

In species having a long period of gestation, e.g., horse (337 days), cow (284 days), monkey (166 days), human, the variations in the length of pregnancy between races or breeds, between individuals of the same race, and between different pregnancies of one individual lead to similar conclusions to those arrived at in the case of the rabbit. In a group of 4083 pregnancies in mares in various parts of central Europe (68), the mean gestation period was 337 days, while the extremes ranged from 264 days to 420 days, the shortest pregnancies being recorded in certain Prussian stock. In a single breed, Rhenish-Belgian, a series of 1000 mares had a mean gestation period of 333 days, the shortest being 304 days and the longest 354 days. In cows, in a series of more than 3000 pregnancies the mean gestation period was 284 days, ranging from 210 to 335 days (68). In this species, according to Hammond (31), the variation in length of the estrous cycle may be normally about 5 days in a cycle which ranges from 18 to 23 days. In 30 pregnancies in rhesus monkeys, Hartman (36) found that the average length of pregnancy was 166 days from the time of copulation; extremes ranged between 146 and 180 days, viable offspring being delivered in all instances. In the human, Hotelling and Hotelling (41) found the mean duration of pregnancy was 281 days in the white race. The shortest duration was 194 days and the longest 317 days in the series under consideration, which thus includes approximately the time from the onset of viability to the end of the period of postmaturity. The differences between the average pregnancy durations of individuals were significantly greater than the differences between the various pregnancies of each individual. No effect was noted between first and later pregnancies, or with respect to the age of the mother. Correlation between the length of pregnancy and the length of the menstrual cycle has been noted in the human (83).

In summary, variations in the gestation periods of a species are such that the average gestation period may approximate a multiple of the cycle, the series of pregnancies under consideration including extreme cases which range from the beginning of viability to the end of the period of postmaturity. Between different breeds or races of one species, the average gestation period may be different. Between different individuals of a breed or race, the average gestation period may be different.

Between various pregnancies of one individual, there may be less difference in duration than between the average durations of pregnancies of various individuals within the same race.

The actual number of days during which the fetus may be held within the uterus after attaining the state of viability and still survive after birth, i.e., the interval from the beginning of prematurity to the end of postmaturity, must be borne in mind in the evaluation of variations in the gestation periods, especially when species with widely different periods of gestation are considered, e.g., opossum (13 days) (37) and the human. The magnitude of the variations must be estimated not merely in actual days, but also with reference to certain facts of embryonic development. With regard to the latter factor, it is significant that the entire pregnancy (13 days) has been completed in the opossum by the time implantation is beginning in the cat. In fact the development of the opossum embryo from the primitive streak stage until birth, occurs in 5½ days, a briefer period than that elapsing in the rabbit from the onset of viability until the end of postmaturity (7 days). Because the standard deviation from the average gestation period is 1 day in the rabbit, or 11 days in the human, a result which would be disastrous for the birth of living offspring in the opossum, one may not conclude that these variations are of such magnitude as to disprove a relation between the length of gestation and the length of the cycle in other species. In fact, from the standpoint of embryonic development a variation of 2 days in the time of birth of a fetus which is capable of survival if extruded at any time over a period of 7 days (rabbit) or a variation of 3 weeks in the time of birth of the human fetus which is capable of survival if born at any time within a period of 3 months, affords evidence of a timing mechanism, the precision of which is of the same order as that which characterizes the sexual cycle.

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